Nucleic acid linkers and their use in gene synthesis

Publication number: EP1314783
Publication date: 2003-05-28
Inventor: SCHATZ OCTAVIAN DR (DE); O'CONNELL TIMOTHY DR (DE)
Applicant: SLONING BIOTECHNOLOGY GMBH (DE)
Classification:
- international: ***C12N15/10; C12N15/66; C12P19/34;*** **C12N15/10; C12N15/66; C12P19/00;** (IPC1-7): C12N15/10; C12N15/66; C12P19/34
- European: C12N15/10C100; C12N15/10; C12N15/66; C12P19/34
Application number: EP20010127864 20011122
Priority number(s): EP20010127864 20011122

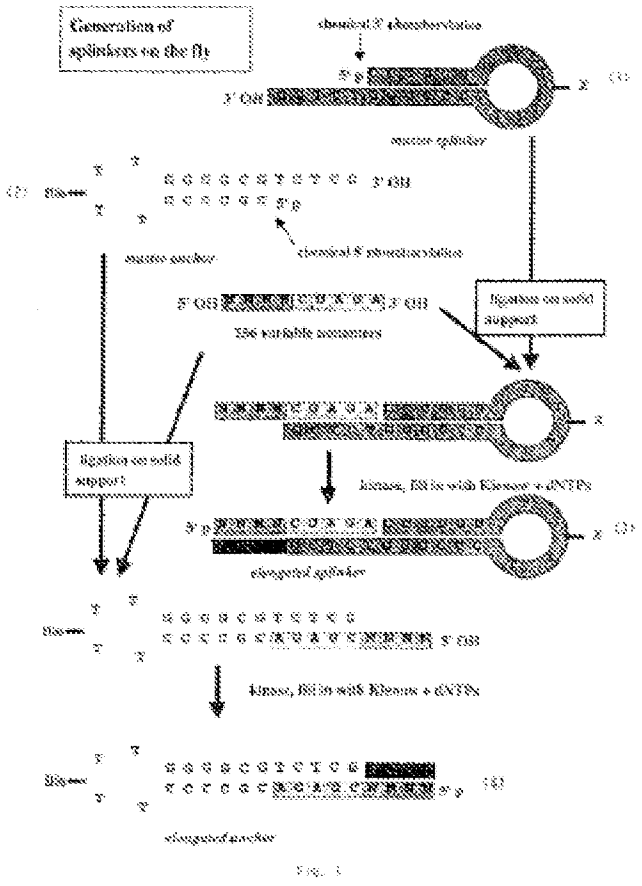
Also published as:
WO03044193 (A3)
WO03044193 (A2)
CA2468235 (A1)
AU2002352108 (A1)

Cited documents:
WO0075368
WO0175180
WO0161036
WO9612014
EP0245130
more >>

Report a data error here

Abstract of **EP1314783**

Single-stranded nucleic acid (I), for use in preparation of nucleic acid (NA) comprising a part (A) comprising the sequence of a recognition site for a type IIS restriction enzyme, or its fragments or complement, and a part (B) that is a defined sequence of nucleotides, is new. <?? >Independent claims are also included for: <??> (1) a library of many (I); <??>(2) method for preparing nucleic acid (NA) using (I); <??>(3) kit for preparation of NA containing the library of (1); <??>(4) methods for enzymatic preparation of a partly double-stranded oligonucleotide (ON) with a 3 nucleotide overhang; <??>(5) several methods for preparing NA, or a group of NA molecules, using ON; and <??>(6) method for amplifying a ligation product, produced during a sloning process.



Result Page

[0001] The present invention concerns einzelsträngige and doppelsträngige nucleic acid molecules to the use in a method to the preparation of a nucleic acid, a method to the preparation of a nucleic acid as well as a kit to the making of a nucleic acid.

[0002] The synthesis of nucleic acids finds various uses in the modern biotechnology. Beside the synthesis of comparatively short nucleic acids such as Oligonukleotiden thereby the synthesis of several Kilobasen large nucleic acids stands increasingly in the foreground. The methods used with it usually use for each gene different synthetic manufactured Oligonukleotide of typical way 40 to 100 nucleotides length as basic modules. Due to the multiplicity of the necessary reaction steps these contain, despite comparatively high coupling efficiencies of approx. 98-99% per step both abort products and false sequences, which are unfavorable for the quality of nucleic acids which can be synthesized. Such errors are above all unfavorable if nucleic acid which can be synthesized represents a coding sequence and it comes due to the offset of the read raster to shortened Transkriptions or translation products. Therefore the Oligonukleotidbausteine must be up-cleaned additionally, in order to reduce these errors to a reasonable amount, since otherwise complex gene syntheses are practically impossible.

[0003] To in the conditions the technology admitted methods counts for example the so-called ?gap filling? - methods, in which a multiplicity of Oligonukleotiden synthesizes, to be up-cleaned and afterwards in pairs or in sub-groups be hybridized. After the synthesis of the respective Gegenstränge by means of a Klenow polymerase reaction the individual fragments are ligiert with one another. The Ligationsprodukte developed in such a way can be kloniert either as partial fragments or be hybridized first with Oligonukleotidprimern lying outside and be amplifiziert in a polymerase nuclear chain reaction (PCR). Alternatively complementary Oligonukleotide can be hybridized with one another in the context of the so-called cartridge method and in such a way received gene fragments by enzymatic or chemical Ligation be linked. After Aufreinigung and/or Klonierung these can be joined to larger gene sections. Both methods are connected, so with disadvantages to z. B. under the errors in the Klenow polymerase reaction or in the polymerase nuclear chain reaction, increasingly more frequently arising with increasing length of nucleic acid which can be synthesized.

[0004] The moreover one methods are well-known in the state of the art, with which Oligonukleotide in a solid phase synthesis are linked with one another, in order to develop larger nucleic acids. Thus for example the international patent application describes WHERE 99/47536 a recursive method, in which einzelsträngige Oligo nucleotides in a defined orientation are ligiert sequenziell to an immobilized starter molecule. The high number of single steps necessary for larger gene syntheses with this method, which lead principle conditionally to small yields as well as to the enrichment of false sequences, is unfavorable. Besides all Oligonukleotide for each synthesis, used for the gene synthesis, must before again be synthesized. A standardisation of this method is only limited possible because of the associated enormous technical expenditure therefore.

top

[0005] The international patent application WHERE 00/75368 describes a combinatorial solid phase synthesis, in which Oligonukleotide, which a recognition sequence for a type contains IIS Restriktionsenzym, with further doppelsträngigen Oligonukleotiden, which are ligiert a recognition sequence for another restriction enzyme contained by the type IIS, doppelsträngige in parallel reaction beginnings, and the Ligationsprodukte afterwards with a type IIS Restriktionsenzym are split. Thus iterative a defined nucleic acid is developed by multiple repeating. This method has the advantage opposite other methods which are based on Ligation of Oligonukleotiden that the used Oligonukleotide contains recognition sequences for different type IIS Restriktionsenzyme, which permits an sequence-independent combination of parallel ligierter partial fragments. Arbitrary partial fragments can be generated from a standardized nucleic acid library with a defined number of elements. The number this library of developing elements depends thereby of the length of the overhangs produced by the respective restriction enzyme. With an overhang of for example four nucleotides, a complete library consists of altogether 65,536 elements. This number results from the number of sequence variants, those in the case of a length of the overhang of four nucleotides (4< 4> = 256) exist, multiplied by the number of sequence variants for the four directly bordering nucleotides, which form the overhang with the next Ligationsschritt (4< 4> x 4< 4> = 65,536).

[0006] Although this method permits an sequence-independent linkage of arbitrary parallel manufactured gene fragments and thus supplies the basis for an automation, the number of necessary Oligonukleotide is still comparatively high for the structure of an appropriate library, which in the context of the synthesis one accesses. A further aspect, which it applies to consider thereby, is the length of the Oligonukleotide, which are used in a such method. (typical way about 20 to 40 nucleotides). Due to its a complete library contains 40 APPROX 2,6 millions of 65,536 x the case described by Oligonukleotiden in altogether Nucleotides.

[0007] Despite with the method after WHERE 00/75368 directly connected the advantages therefore further the need consists to make a method available to the synthesis of nucleic acids and agent to the lead-through of the same in the state of the art which complexity smaller on a Oligonukleotid library falls back. In particular the expenditure is to be reduced for the production of a complete library, particularly regarding the number of nucleotides necessary for it,

opposite the state of the art clearly without thereby disadvantages like a smaller Ligationseffizienz (e.g. to have to take with the use of overhangs of only or two nucleotides length) in purchase. Thereby a further task of the invention a method is to be made available, which permits a still smaller portion of abort and false sequences ensured as well as the simultaneous synthesis of several gene variants.

[0008] According to invention this task in a first aspect solved by a einzelsträngiges nucleic acid molecule to the use in a method to the preparation of a nucleic acid comprising at least
(constant) a part A and (variable) a part B
how

Part A a sequence covers, which corresponds to the sequence of the recognition sequence of a restriction enzyme of the type II S or a part of it or a sequence complementary in addition, and
Part B a defined succession of nucleotides covers.

[0009] In an embodiment it is intended that the restriction enzyme is selected from the group, which covers BpiI, Esp3I, Eco31I, BsaI, BsmBI, BbsI, BspMI, AarI, AceIII, Acc36I, SapI, BtsI, BsrDI, Bse3DI, BciVI, BfuI, BfiII and BmrI.

[0010] In a further embodiment it is intended that the sequence is selected by part A from the group, the SEQ ID NO: 1 to 13 covers.

[0011] In a still further embodiment it is intended that part B exhibits a length of 1, 2, 3, 4, 5, 6 or 7 nucleotides.

[0012] In a second aspect the solve the problem become by a nucleic acid molecule library a comprising multiplicity of the nucleic acid molecules according to invention.

[0013] In an embodiment it is intended that the library of 256 members differentiating with respect to the sequence of part B covers itself, whereby the defined succession of nucleotides of part B exhibits a length of four nucleotides.

[0014] In a further embodiment it is intended that the library covers itself 1024 in the sequence of part B differentiating members, whereby the defined succession of nucleotides of part B exhibits a length of five nucleotides.

[0015] In a still further embodiment it is intended that the library of 4096 members differentiating with respect to the sequence of part B covers itself, whereby the defined succession of nucleotides of part B exhibits a length of six nucleotides.

[0016] In a further embodiment it is intended that the library of 16 members differentiating with respect to the sequence of part B covers itself, whereby the defined succession of nucleotides of part B exhibits a length of two nucleotides.

[0017] Finally it is intended in an embodiment that the library of 64 members differentiating with respect to the sequence of part B covers itself, whereby the defined succession of nucleotides of part B exhibits a length of three nucleotides.

[0018] In a third aspect the solve the problem become by the use at least one of the nucleic acid molecules according to invention and/or one of the nucleic acid molecule libraries according to invention in a method the preparation of nucleic acids, in particular a method the sequenziellen Ligation of Oligonukleotiden in parallel reaction beginnings, which are linked with one another in further steps in sequence-independent way.

[0019] In a fourth aspect the solve the problem become by a method the preparation of a nucleic acid molecule the comprising steps:

a) Makes available a first Oligonukleotids, optionally by means of a modification to a solid phase coupled, whereby the Oligonukleotid a recognition sequence or a part of it covers or complementary a for this sequence for first type IIS Restriktionsenzym, which cut outside of its recognition sequence, and a einzelsträngigen overhang,
b) Adds a einzelsträngigen nucleic acid molecule according to invention to the Oligonukleotid, whereby preferential way the part A of the nucleic acid molecule is essentially complementary to the einzelsträngigen range of the first Oligonukleotids;
c) Ligation of the nucleic acid molecule from step b) with the first Oligonukleotid under construction over-hanging 5 of a ' end;
d) Fills up over-hanging 5 ' - the end;
e) Makes available a second Oligonukleotides, whereby the Oligonukleotid a recognition sequence or a part enclosure of it or a complementary for this sequence for second type IIS Restriktionsenzym, which cut outside of its recognition sequence, and a einzelsträngigen overhang, whereby the recognition sequence of this restriction enzyme is different from the recognition sequence in step of the A) restriction enzyme specified, and
f) Adds a einzelsträngigen nucleic acid molecule according to invention to the Oligonukleotid, whereby preferential way the part A of the nucleic acid molecule is essentially complementary to the einzelsträngigen range of the second Oligonukleotids;
g) Ligation of the nucleic acid molecule of step f) with the second Oligonukleotid;
h) Fills up supernatant 5 ' - the end;
i) Ligation of the Oligonukleotide received from the steps A) to D) and e) to h);
j) Cracking of the Ligationsproduktes with the first or with the second type, received in step i), IIS Restriktionsenzym.

In an embodiment it is intended that in step b) and/or f) a hybridizing between the einzelsträngigen range of the Oligonukleotids with part A of the einzelsträngigen nucleic acid molecule takes place.

[0020] In a further embodiment it is intended that the first Oligonukleotid coupled way before the Ligation in accordance with step i, preferential to a solid phase and) from the solid phase abgespalten becomes.

[0021] In a fifth aspect the solve the problem become by a kit the making of a nucleic acid comprising one of the nucleic acid libraries according to invention or a part of it.

[0022] In an embodiment it is intended that the kit a first Oligonukleotid covers a comprising recognition sequence for first type IIS Restriktionsenzym.

[0023] In a further embodiment it is intended that the kit a far second Oligonukleotid covers a comprising recognition sequence or a part of it or complementary a for this sequence for second type IIS Restriktionsenzym, whereby the second restriction enzyme of the first restriction enzyme is different.

[0024] In a still further embodiment it is intended that at least one is the Oligonukleotide at a solid phase immobilized.

[0025] In a sixth aspect the invention concerns a method to the enzymatic preparation partial doppelsträngigen Oligonukleotids with a 3 nucleotide long overhang, whereby the Oligonukleotid contains a recognition sequence for a restriction enzyme of the type IIS, the comprising steps:

a) Makes available a first partial doppelsträngigen Oligonukleotids, whereby the Oligonukleotid a 3 ' - overhang and a recognition sequence for a restriction enzyme of the type IIS exhibit,
b) Make available to a first group way preferential by einzelsträngigen Oligonukleotiden a comprising part A and a part B, whereby part A complementary to the einzelsträngigen range in step of the A) made available first Oligonukleotids and with all members of the group identically are and part B a length of 3 nucleotides covered, whereby the members of the group differ in part B,
c) Makes available a second partial doppelsträngigen Oligonukleotids, whereby the Oligonukleotid a 3 ' - overhang and a recognition sequence for a restriction enzyme of the type IIS exhibit, whereby the restriction enzyme of the type is different IIS from the restriction enzyme of the type IIS of the Oligonukleotide in step A),
d) Make available to a second group way preferential by einzelsträngigen Oligonukleotiden a comprising part A and a part B, whereby part A complementary to the einzelsträngigen range in step of the A) made available first Oligonukleotids and with all members of the group identically are and part B a length of 3 nucleotides covered, whereby the members of the group differ in part B,
e) Hybridisation and Ligieren of the first Oligonukleotids with in each case a member of the first group of einzelsträngigen Oligonukleotiden, made available made available in step A), in step b),
f) Hybridisation and Ligieren of the second Oligonukleotids with in each case a member of the second group of einzelsträngigen Oligonukleotiden, made available made available in step C), in step D)
g) Fill up the over-hanging 5 ' - ends of the Ligationsprodukte from step e),
h) Fill up the over-hanging 5 ' - ends of the Ligationsprodukte from step f),
i) Ligation of in each case a filled up Ligationsprodukt from step g) with in each case a filled up Ligationsschritt from step h),
j) Cracking of the Ligationsproduktes from step i) with the restriction enzyme of the type IIS, specific to the Oligonukleotid made available in step A)

[0026] In a sieved aspect the invention concerns a method to the enzymatic preparation partial doppelsträngigen Oligonukleotids with a 3 nucleotide long overhang, whereby the Oligonukleotid contains a recognition sequence for a restriction enzyme of the type IIS and of cutting edges of the Oligonukleotids with the restriction enzyme to an overhang with a length different of 3 nucleotides, the comprising steps:

a) Makes available a first partial doppelsträngigen Oligonukleotids, whereby the Oligonukleotid a 3 ' - overhang exhibits and a recognition sequence for a restriction enzyme of the type IIS exhibits,
b) Make available to a first group way preferential by einzelsträngigen Oligonukleotiden a comprising part A and a part B, whereby part A complementary to the einzelsträngigen range in step of the A) made available first Oligonukleotids and with all members of the group identically are and part B a length of 2 nucleotides covered, whereby the members of the group differ in part B,
c) Makes available a second partial doppelsträngigen Oligonukleotids, whereby the Oligonukleotid a 3 ' - overhang exhibits and a recognition sequence for a restriction enzyme of the type IIS exhibits, whereby the restriction enzyme of the type is different IIS from the restriction enzyme of the type IIS of the Oligonukleotide in step A),
d) Make available to a second group way preferential by einzelsträngigen Oligonukleotiden a comprising part A and a part B, whereby part A complementary to the einzelsträngigen range in step of the A) made available first Oligonukleotids and with all members of the group identically are and part B a length of 2 nucleotides covered, whereby the members of the group differ in part B,
e) Hybridisation and Ligieren of the first Oligonukleotids with in each case a member of the first group of einzelsträngigen Oligonukleotiden, made available made available in step A), in step b),
f) Hybridisation and Ligieren of the second Oligonukleotids with in each case a member of the second group of einzelsträngigen Oligonukleotiden, made available made available in step C), in step D)
g) Fill up the over-hanging 5 ' - ends of the Ligationsprodukte from step e),
h) Fill up the over-hanging 5 ' - ends of the Ligationsprodukte from step f),
i) Ligation of in each case a filled up Ligationsprodukt from step g) with in each case a filled up Ligationsschritt from step h),
j) Cracking of the Ligationsproduktes from step i) with the restriction enzyme of the type IIS, specific to the Oligonukleotid made available in step A)

[0027] In a respected aspect the invention concerns a method to the preparation of a nucleic acid molecule of the comprising steps

a) Make a Oligonukleotids, that available is manufactured by the following steps:

aa) making available partial doppelsträngigen Oligonukleotids with 5 ' - overhang, which a recognition sequence for a TypIIS contains restriction enzyme, which cuts outside of its recognition sequence, and a modification carries, which permits a coupling to a solid matrix, whereby the 5 ' - an overhang a length of 3 nucleotides covers,
off) addition of a further, at least partial doppelsträngigen Oligonukleotids with 5 ' - overhang and another recognition sequence for a TypIIS restriction enzyme, which cuts outside of its recognition sequence, than in step aa), whereby the

5 ' - an overhang a length of 3 nucleotides covers,
AC) Ligation of the Oligonukleotide from step aa) and off) in orientation specified by the blocking that not to ligierenden ends,
ad) removing not spent reactant as well as enzymes,
ae) cracking of the Ligationsprodukts from step AC) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the nucleic acid sequence of the Oligonukleotids from step off) takes place,
af) separating of the reaction mixture of in step ae) received elongated Oligonukleotid from step aa),
AG) optionally at least unique repeating of the steps off) to af),

b) Make a further Oligonukleotids, that available is manufactured by the steps:

ba) making available partial doppelsträngigen Oligonukleotids with 5 ' - overhang, which a recognition sequence for a TypIIS contains restriction enzyme, which cuts outside of its recognition sequence, and a modification carries, which permits a coupling to a solid matrix, whereby the 5 ' - an overhang a length of 3 nucleotides covers,
bb) addition of a further, at least partial doppelsträngigen Oligonukleotids with 5 ' - overhang and with another recognition sequence for a TypIIS restriction enzyme, which cuts outside of its recognition sequence, than in step ba), whereby the 5 ' - an overhang a length of 3 nucleotides covers,
UC) Ligation of the Oligonukleotide from step ba) and bb) in orientation specified by the blocking that not to ligierenden ends,
bd) removing not spent reactant as well as enzymes,
) cracking of the Ligationsproduktes from step UC) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the Oligonukleotid in step bb) takes place,
BF) Separating of the so elongated nucleic acid molecule of the reaction mixture,
bg) optionally at least unique repeating of the steps bb) to), whereby following the last Ligation in step UC) and removing not spent reactant as well as enzymes the Ligationsprodukt with a TypIIS restriction enzyme is cut, whereby the cracking in the Oligonukleotid from step ba) takes place,

c) Ligation of the Oligonukleotide from step A) and b) in orientation specified by the blocking that not to ligierenden ends,
d) Do not remove spent reactant as well as enzymes,
e) Cracking of the Ligationsproduktes from step C) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the Oligonukleotid from step A) or b takes place),
f) Separating of the so elongated nucleic acid molecule of the reaction mixture,
whereby the Oligonukleotid from step) the recognition sequence of a restriction enzyme of the type IIS exhibits, which three nucleotides are repeated a long overhang produced, as long as the steps off) to ae) and the Oligonukleotid from step) the recognition sequence of a restriction enzyme of the type IIS exhibit, which produces another than for three nucleotide a long overhang, when last going through the steps off) to ae) and/or
the Oligonukleotid of step bb) the recognition sequence of a restriction enzyme of the type IIS exhibits, which three nucleotides are repeated a long overhang produced, as long as the steps bb) to) and the Oligonukleotid of step bb) the recognition sequence of a restriction enzyme of the type IIS exhibit, which produces another than for three nucleotide a long overhang, when last going through the steps bb) to).

[0028] In a ninth aspect the invention concerns a method to the preparation of a group of nucleic acid molecules of the comprising steps

a) Make a Oligonukleotids, that available is manufactured by the following steps:

aa) making a Oligonukleotids available, which a recognition sequence for a TypIIS contains restriction enzyme, which cuts outside of its recognition sequence, and a modification carries, which permits a coupling to a solid matrix,
Coupling of the Oligonukleotids to the solid matrix
off) addition of a further, at least partial doppelsträngigen Oligonukleotids with another recognition sequence for a TypIIS restriction enzyme, which cuts outside of its recognition sequence, than in step aa),
AC) Ligation of the Oligonukleotide from step aa) and off) in orientation specified by the blocking that not to ligierenden ends,
ad) removing not spent reactant as well as enzymes,
ae) cracking of the Ligationsprodukts from step AC) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the nucleic acid sequence of the Oligonukleotids from step off) takes place,
af) separating of the reaction mixture of in step ae) received elongated Oligonukleotid from step aa),
AG) optionally at least unique repeating of the steps off) to af),

b) Make a further Oligonukleotids, that available is manufactured by the steps:

ba) making a Oligonukleotids available, which a recognition sequence for a TypIIS contains restriction enzyme, which cuts outside of its recognition sequence, and, the one coupling carries a modification to a solid matrix permitted with an end to a solid matrix,
Coupling of the Oligonukleotids to the solid matrix,
bb) addition of a further, at least partial doppelsträngigen Oligonukleotids with another recognition sequence for a TypIIS restriction enzyme, which cuts outside of its recognition sequence, than in step ba),
UC) Ligation of the Oligonukleotide from step ba) and bb) in orientation specified by the blocking that not to ligierenden ends,
bd) removing not spent reactant as well as enzymes,
) cracking of the Ligationsproduktes from step UC) with a TypIIS restriction enzyme, which cuts outside of its recognition

sequence, whereby the cracking in the Oligonukleotid in step bb) takes place,
BF) Separating of the so elongated nucleic acid molecule of the reaction mixture,
bg) optionally at least unique repeating of the steps bb) to BF), whereby following the last Ligation in step UC) and removing not spent reactant as well as enzymes the Ligationspordukt with a TypIIS restriction enzyme is cut, whereby the cracking in the Oligonukleotid from step ba) takes place,

c) Ligation of the Oligonukleotide from step A) and b) in orientation specified by the blocking that not to ligierenden ends,
d) Do not remove spent reactant as well as enzymes,
e) Cracking of the Ligationsproduktes from step C) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the Oligonukleotid from step A) or b takes place),
f) Separating of the so elongated nucleic acid molecule of the reaction mixture,
whereby when last repeating the steps off) as far as af) the Oligonukleotid admitted in step off) a modification carries, the one coupling to a solid matrix permitted and
after last repeating of the steps) to af) as step ah) the Ligationsprodukt from step AC) with a TypIIS restriction enzyme it is cut off which cuts the Spaltungsprodukt outside of its recognition sequence, whereby the cracking in the nucleic acid sequence of the Oligonukleotids from step aa) takes place and by the Oligonukleotid coupled at the solid matrix is set free
and the set free fission product into at least two reaction beginnings is divided.

[0029] In a tenth aspect the solve the problem become by a method the preparation of a nucleic acid molecule the comprising steps

a) Make a Oligonukleotids, that available is manufactured by the following steps:

aa) making a Oligonukleotids available, which a recognition sequence for a TypIIS contains restriction enzyme, which cuts outside of its recognition sequence, and a modification carries, which permits a coupling to a solid matrix,
Coupling of the Oligonukleotids to the solid matrix
off) addition of a further, at least partial doppelsträngigen Oligonukleotids with another recognition sequence for a TypIIS restriction enzyme, which cuts outside of its recognition sequence, than in step aa),
AC) Ligation of the Oligonukleotide from step aa) and off) in orientation specified by the blocking that not to ligierenden ends,
ad) removing not spent reactant as well as enzymes,
ae) cracking of the Ligationsprodukts from step AC) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the nucleic acid sequence of the Oligonukleotids from step off) takes place,
af) separating of the reaction mixture of in step ae) received elongated Oligonukleotid from step aa),
AG) optionally at least unique repeating of the steps off) to af),

b) Make a further Oligonukleotids, that available is manufactured by the steps:

ba) making a Oligonukleotids available, which a recognition sequence for a TypIIS contains restriction enzyme, which cuts outside of its recognition sequence, and, the one coupling carries a modification to a solid matrix permitted with an end to a solid matrix,
Coupling of the Oligonukleotids to the solid matrix,
bb) addition of a further, at least partial doppelsträngigen Oligonukleotids with another recognition sequence for a TypIIS restriction enzyme, which cuts outside of its recognition sequence, than in step ba),
UC) Ligation of the Oligonukleotide from step ba) and bb) in orientation specified by the blocking that not to ligierenden ends,
bd) removing not spent reactant as well as enzymes,
) cracking of the Ligationsproduktes from step UC) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the Oligonukleotid in step bb) takes place,
BF) Separating of the so elongated nucleic acid molecule of the reaction mixture,
bg) optionally at least unique repeating of the steps bb) to BF), whereby following the last Ligation in step UC) and removing not spent reactant as well as enzymes the Ligationspordukt with a TypIIS restriction enzyme is cut, whereby the cracking in the Oligonukleotid from step ba) takes place,

c) Ligation of the Oligonukleotide from step A) and b) in orientation specified by the blocking that not to ligierenden ends,
d) Do not remove spent reactant as well as enzymes,
e) Cracking of the Ligationsproduktes from step C) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the Oligonukleotid from step A) or b takes place),
f) Separating of the so elongated nucleic acid molecule of the reaction mixture,
whereby when last repeating the steps off) as far as af) the Oligonukleotid admitted in step off) a modification carries, which permits a coupling to a solid matrix.

[0030] In a elften aspect the task solved according to invention by a method to the preparation of a nucleic acid molecule of the comprising steps

a) Make a Oligonukleotids, that available is manufactured by the following steps:

aa) making a Oligonukleotids available, which a recognition sequence for a TypIIS contains restriction enzyme, which cuts outside of its recognition sequence,
off) addition of a further, at least partial doppelsträngigen Oligonukleotids with another recognition sequence for a TypIIS restriction enzyme, which cuts outside of its recognition sequence, when in step aa) and a modification carries,

which permits a coupling to a solid matrix,
AC) Ligation of the Oligonukleotide from step aa) and off) in orientation specified by the blocking that not to ligierenden ends,
ad) optionally removing and/or inactivating not spent reactant as well as enzymes,
ae) cracking of the Ligationsprodukts from step AC) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the nucleic acid sequence of the Oligonukleotids from step off) takes place,
af) separating no modification of basic fission product from step ae) of the reaction mixture,
AG) optionally at least unique repeating of the steps off) to af),

b) Make a further Oligonukleotids, that available is manufactured by the steps:

ba) making a Oligonukleotids available, which a recognition sequence for a TypIIS contains restriction enzyme,
bb) addition of a further, at least partial doppelsträngigen Oligonukleotids with another recognition sequence for a TypIIS restriction enzyme, which cuts outside of its recognition sequence, when in step ba) and a modification carries, which permits a coupling to a solid matrix,
UC) Ligation of the Oligonukleotide from step ba) and bb) in orientation specified by the blocking that not to ligierenden ends,
bd) optionally removing and/or inactivating not spent reactant as well as enzymes,
) cracking of the Ligationsproduktes from step UC) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the Oligonukleotid in step bb) takes place,
BF) Separating no modification of basic fission product from step) the reaction mixture,
bg) optionally at least unique repeating of the steps bb) to BF), whereby following the last Ligation in step UC) and removing and/or inactivating spent reactant as well as enzymes the Ligationspordukt with a TypIIS restriction enzyme is not cut, whereby the cracking in the Oligonukleotid from step ba) takes place,

c) Ligation of the Oligonukleotide from step A) and b) in orientation specified by the blocking that not to ligierenden ends,
d) Removing and/or inactivating not spent reactant as well as enzymes,
e) Cracking of the Ligationsproduktes from step C) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the Oligonukleotid from step A) or b takes place),
f) Separating of the so elongated nucleic acid molecule of the reaction mixture,

[0031] In a twelfth aspect the task solved according to invention by a method to the preparation of a nucleic acid molecule of the comprising steps

a) Make a Oligonukleotids, that available is manufactured by the following steps:

aa) making a Oligonukleotids available, which a recognition sequence for a TypIIS contains restriction enzyme, which cuts outside of its recognition sequence, and a modification carries, which permits a coupling to a solid matrix,
Coupling of the Oligonukleotids to the solid matrix
off) addition of a further, at least partial doppelsträngigen Oligonukleotids with another recognition sequence for a TypIIS restriction enzyme, which cuts outside of its recognition sequence, than in step aa),
AC) Ligation of the Oligonukleotide from step aa) and off) in orientation specified by the blocking that not to ligierenden ends,
ad) removing not spent reactant as well as enzymes,
ae) cracking of the Ligationsprodukts from step AC) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the nucleic acid sequence of the Oligonukleotids from step off) takes place,
af) separating of the reaction mixture of in step ae) received elongated Oligonukleotid from step aa),
AG) optionally at least unique repeating of the steps off) to af),

b) Make a further Oligonukleotids, that available is manufactured by the steps:

ba) making a Oligonukleotids available, which a recognition sequence for a TypIIS contains restriction enzyme, which cuts outside of its recognition sequence, and, the one coupling carries a modification to a solid matrix permitted with an end to a solid matrix,
Coupling of the Oligonukleotids to the solid matrix,
bb) addition of a further, at least partial doppelsträngigen Oligonukleotids with another recognition sequence for a TypIIS restriction enzyme, which cuts outside of its recognition sequence, than in step ba),
UC) Ligation of the Oligonukleotide from step ba) and bb) in orientation specified by the blocking that not to ligierenden ends,
bd) removing not spent reactant as well as enzymes,
) cracking of the Ligationsproduktes from step UC) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the Oligonukleotid in step bb) takes place,
BF) Separating of the so elongated nucleic acid molecule of the reaction mixture,
bg) optionally at least unique repeating of the steps bb) to BF), whereby following the last Ligation in step UC) and removing not spent reactant as well as enzymes the Ligationspordukt with a TypIIS restriction enzyme is cut, whereby the cracking in the Oligonukleotid from step ba) takes place,

c) Ligation of the Oligonukleotide from step A) and b) in orientation specified by the blocking that not to ligierenden ends,
d) Do not remove spent reactant as well as enzymes,
e) Cracking of the Ligationsproduktes from step C) with a TypIIS restriction enzyme, which cuts outside of its recognition

sequence, whereby the cracking in the Oligonukleotid from step A) or b takes place),
f) Separating of the so elongated nucleic acid molecule of the reaction mixture,
how
the Oligonukleotid from step aa) and/or the Oligonukleotid from step off) at least a Methylierung exhibit, whereby after at least unique going through of the steps aa) to af) at the Oligonukleotid based on the Oligonukleotid from step aa) at least a part of a recognition sequence of a restriction enzyme by the TypIIS one trained, that in the Ligation with a further Oligonukleotid in accordance with step ae) completed and the Methylierung a cracking of the Ligationsproduktes in such a way manufactured using this recognition place one prevents and/or
the Oligonukleotid from step aa) and/or the Oligonukleotid from step off) at least a Methylierung exhibit, whereby after at least unique going through of the steps aa) to af) at the Oligonukleotid based on the Oligonukleotid from step off) at least a part of a recognition sequence of a restriction enzyme by the TypIIS one trained, that in the Ligation with a further Oligonukleotid in accordance with step ae) completed and the Methylierung a cracking of the Ligationsproduktes in such a way manufactured using this recognition place one prevents and/or
the Oligonukleotid from step ba) and/or the Oligonukleotid from step bb) at least a Methylierung exhibit, whereby after at least unique going through of the steps ba) to BF) at the Oligonukleotid based on the Oligonukleotid from step ba) at least a part of a recognition sequence of a restriction enzyme by the TypIIS one trained, that in the Ligation with a further Oligonukleotid in accordance with step) completed and the Methylierung a cracking of the Ligationsproduktes in such a way manufactured using this recognition place one prevents and/or
the Oligonukleotid from step ba) and/or the Oligonukleotid from step bb) at least a Methylierung exhibit, whereby after at least unique going through of the steps ba) to BF) at the Oligonukleotid based on the Oligonukleotid from step bb) at least a part of a recognition sequence of a restriction enzyme by the TypIIS one trained, in the Ligation with a further Oligonukleotid in accordance with step) completed and the Methylierung the cracking of the Ligationsproduktes in such a way manufactured using this recognition place one prevents.

[0032] In a three tenth aspect the task solved according to invention by a method for amplification one in the context of a Sloning method enstehenden Ligationsproduktes, whereby the method covers the following steps:

a) Make available a Ligationsproduktes;
b) Make available one for the Oligonukleotid in accordance with step aa) and/or off) the Sloning method at least partial of complementary primer,
c) Make available one for the Oligonukleotid in accordance with step off) and/or bb) the Sloning method at least partial of complementary primer,
d) Hybridisation at least one that primer with the Ligationsprodukt;
e) Accomplish a polymerase nuclear chain reaction using the primer hybridized to the Ligationsprodukt.

[0033] Further embodiments of the different aspects of the present invention result from the Unteransprüchen.

[0034] The present invention is the basis the surprising realization that with the method after WHERE 00/75368 possible sequence-independent linkage of arbitrary Oligoukleotide and thus the synthesis of arbitrary nucleic acids can be still further improved, because by the smaller complexity of the Oligonukleotidbibliothek (like it after the method of the present application to be generated can) a higher standardisation of the Oligonukleotide contained in it is possible. In particular a higher yield and a larger purity can be achieved by the Verkürzung of the Oligonukleotide possible after the available method, whereby the accuracy of the gene synthesis can be again increased. In the method after WHERE 00/75368 two different classes become by Oligonukleotiden used, whereby the two called themselves there as Anchor and Splinker Oligonukleotid classes by the presence of two different recognition sequences for restriction enzymes of the type IIS and the individual elements within the Oligonukleotidklasse with respect to the sequence of the respective overhang differentiate. In order to be able to manufacture arbitrary genes after this method, a library from Oligonukleotiden must stand to the order, which contains all possible sequence variants of the Oligonukleotide lying to reason. According to the present invention knows the Anchor and Splinker Oligonukleotide needed in each case using altogether three standardized elements if necessary in a modification of the 00/75368 method described in the application WHERE to be manufactured. With the three elements it concerns to two classes of Oligonukleotiden, which essentially by the presence of at least in each case a recognition sequence (or the sequence complementary in addition) for a restriction enzyme of the type IIS differ, and a einzelsträngiges Oligonukleotid called herein in the following also left one.

[0035] Restriction enzymes of the type IIS are are characterised by the fact that they step with two discrete places of a doppelsträngigen DNA into reciprocal effect. One of the two places is the recognition place, which exhibits typical way a length from 4 to 7 pairs of cousins. The other place is the splitting place, which is usually for 1 to 20 pairs of cousins distant from the recognition place. The recognition places of the restriction enzymes are either complete or partial asymmetric.

[0036] The two classes of at least in each case one recognition sequence Oligonukleotiden exhibiting for a restriction enzyme of the type IIS (or the sequence complementary in addition) cover preferential way in each case the following structural components in 3 ' - 5 ' - direction: a einzelsträngigen range, a doppelsträngigen range and, optionally, a loop (English. Loop). This secondary structure is formed in consequence of the primary structure of appropriate einzelsträngigen nucleic acid.

[0037] Dem einzelsträngigen Bereich kommt insoweit eine besondere Bedeutung bei, als dass dieser vollständig oder teilweise die Erkennungssequenz für ein Restriktionsenzym vom Typ IIS darstellt oder enthält. Alternatively the einzelsträngige range can cover also a sequence, those is more complementary to the complete recognition sequence for the restriction enzyme of the type IIS or a part the same. The minimum length of the einzelsträngigen range can be thereby an individual nucleotide. The maximum length of this range is in principle not limited thereby, however it is preferentially, if this does not contain further nucleotides apart from the recognition sequence, there itself thereby the length of the Oligonukleotids in the long run only unnecessarily elongated, which is connected for a higher risk by false sequences with a increased synthesis expenditure and with it accompanying. On the other hand the used overhang should permit a stable hybridizing with the einzelsträngigen left one. As optimal therefore lengths between 3 and 7 nucleotides are to be regarded.

[0038] The doppelsträngige range can develop from the back folding of the Oligonukleotids on itself. The length of the doppelsträngigen range amounts to preferential way three to nine nucleotides. Fundamental is specific succession nucleotides in this range arbitrary, if at least under the conditions of the nucleic acid synthesis, with which the Oligonukleotid is to be used, a stable hybridizing between the complementary nucleotides of the Oligonukleotids takes place. The construction of office mating is preferential in consequence of the increased stability of an office mating in relation to an RK mating. If TypIIS of restriction enzymes come also far distant interfaces to the inset, the recognition sequences for these enzymes can both completely and to be partly contained in the doppelsträngigen range.

[0039] The loop of the Oligonukleotids can be formed from any succession by nucleotides. With the selection of the succession it applies to ensure however that no reciprocal effect with other sequences takes place and is disturbed thus the construction of the loop or the others the Oligonukleotid developing (secondary) structures. Pyrimidines are preferred and completely particularly thymus-serve used, since these are relatively small and are stable the developing loop structure. Cytosine is received with Guanosin more stable base pairing, whereby the construction of alternative secondary structures is favoured. The use of preferential way four pyrimidines and prefers thymus serving arises from the fact that the hoop stress with less than 4 nucleotides too largely will (whereby the adjacent doppelsträngigen regions to be dissolved to be able). More than 4 nucleotides have however no considerable effect on the hoop stress and would be redundant therefore.

[0040] Those managing described class of Oligonukleotiden is called herein one-piece or complementary Oligonukleotid. Alternative a for this class of Oligonukleotiden, which exhibits however the same function, in particular in the context of its use to the towards or nucleic acid synthesis, is characterised by the fact that the loop is missing. This class of Oligonukleotiden can be produced by it that two einzelsträngige Oligonukleotide is with one another hybridized, whereby it comes to the construction of the doppelsträngigen and the einzelsträngigen range. In consequence of the absence of the loop einzelsträngigen Oligonukleotide which can be hybridized is both regarding the sequence and the modification at the 3 ' - and/or during the selection and arrangement of the two. 5 ' - to employ end a set of considerations and/or. To seize measures. As in the following it is still described must be ensured by two-piece Oligonukleotid with this form that it comes to no incorrect mating with that left ones. Further it must be ensured that the ends of the two hybridized single strands lying exposed due to the absence of the loop do not represent substrate for a polymerase or a ligase. Dies kann beispielsweise dadurch gewährleistet werden, dass eine Aminoverbindung, ein Succinylester, ein Fluoreszenzfarbstoff oder ein Digoxigeninrest an die endständigen 5' bzw. 3 ' groups of phosphates one couples.

[0041] Both alternatives of Oligonukleotidklassen, i.e. both the complementary form as well as the two-piece form of the Oligonukleotide, can a modification have, which permits it to couple the Oligonukleotid to a solid phase. In case of the complementary form this modification of preferential way takes place in the range of the loop. With this modification it is ensured that the Oligonukleotid or this a comprising nucleic acid can be separated from other connections. The modification can take place via the specialists measures well-known on the field. Beispielhafte Modifikationen sind der Einbau niedermolekularer Verbindungen wie Biotin, Digoxigenin, Fluoresceinisothiocyanat (FITC), Aminoverbindungen oder Succinylester. Die Oberfläche wird in der Folge Moleküle aufweisen, die eine in der Regel spezifische Wechselwirkung mit der Modifikation zu Immobilisierungszwecken erlaubt.

[0042] That left ones as the third standardized element is a Oligonukleotid chemically regarded likewise. Der Linker besteht grundsätzlich aus zwei Teilsequenzen. The first (constant) partial sequence, herein also as part A designation, covers at least the recognition sequence of a restriction enzyme of the type IIS or a part of it. Alternatively part A can cover the sequence or a part of, it complementary to the recognition sequence of the restriction enzyme of the type IIS. The second (variable) partial sequence of the Linkers, herein also as part B designation, represents any, but defined consequence of nucleotides. The specific arrangement of the constant part A of the Linkers depends thereby on the respective restriction enzymes on the type IIS, those in the context of the synthesis procedure to be used and/or. to those the two classes of Oligonukleotiden turn off (usually these completely complementary to the einzelsträngigen range at least one the Oligonukleotide described above is).

[0043] In the following the arrangement of a Oligonukleotids and the appropriate Linkers on the assumption is represented that the einzelsträngige range of the Oligonukleotids covers the recognition sequence of the restriction enzyme of the type IIS completely. Due to the property of these restriction enzymes that the interface lies outside of the recognition place, i.e. the recognition place by the enzymatic activity is not destroyed, and beyond that the cut of the restriction enzyme taken place in a defined distance independently of its recognition place from the sequence which can be cut, can be arranged that left ones in such a way that the constant part is more complementary A to the recognition sequence of the restriction enzyme, which trains the einzelsträngigen range of the Oligonukleotids. In consequence of this Komplementarität a hybridizing can take place from Oligonukleotid and left ones. After that covers left ones apart from part A still part B, part B of the Linkers forms an overhang or a supernatant end after hybridizing with the Oligonukleotid. The same structure from Oligonukleotid and left ones is formed, if the Oligonukleotid within its einzelsträngigen range exhibits a sequence, which is more complementary for the recognition sequence of the restriction enzyme and part A of the Linkers the recognition sequence of the restriction enzyme represents. It is not necessary thereby that either the einzelsträngige range of the Oligonukleotids or the constant part A of the Linkers the complete recognition sequence and/or. their complementary sequence covers. Rather it is also in the context of the invention, if the recognition sequence or the sequence of the restriction enzyme complementary in addition is altogether trained by parts of the doppelsträngigen range and the einzelsträngigen range. In this case for example part A of the Linkers will cover only the part of the recognition sequence of the Restriktionsenyms, which is more complementary to in the einzelsträngigen range of the Oligonukleotids contained the part.

[0044] The length of part B of the Linkers is determined more exactly by the restriction enzyme used in each case and by the length of the overhang produced by it. The following table 1 shows an overview of different restriction enzymes of the type IIS with their recognition sequences and the produced overhangs. The table represents the pairs of restriction enzymes, which are used favourable way in the nucleic acid synthesis procedure according to invention as well as the standardized elements.
< tb> < TABLE> Id=Tabelle 1: Columns=4
< tb> : Exemplary arrangement of Oligonukleotid 1 and Oligonukleotid 2 as well as the Linkers according to invention as

a function of the specifically used pair of restriction enzymes on the type IIS.
< tb>
< tb> Head Col 1: Restriction of pair of enzymes
< tb> Head Col 2: Oligonukleotid 1 (5 ' - 3 ')
< tb> Head Col 3: Oligonukleotid 2 (5 ' - 3 ')
< tb> Head Col 4: Left (5 ' - 3 ')
< tb> Eco31I/Esp3I< SEP> CGN1-9X1-9N' 1-9CGTCTCN
(SEQ. Identification No. 14) < SEP> CCN1-9X1-9N'1-9GGTCTCN (SEQ. Identification No. 16) < SEP> NNNNN' GAGA
(SEQ. Identification No. 18)
< tb> BbsI/Acc36I< SEP> TTCN1-9X1-9N'1-9GAAGACNN
(SEQ. Identification No. 15) < SEP> CAGGTN1-9X1-9N'1-9ACCTGCN4 (SEQ. Identification No. 17) < SEP> A) NNNNN'2
GTC (SEQ ID No. 19)
b) NNNNN'4 G (SEQ ID No. 20)
< tb> Eco31I/Esp3I
(bipartite) < SEP> N1-9CGTCTCN
(SEQ. ID.No.21)
CGN'1-9
(SEQ. ID.No.22) < SEP> CCN'1-9
(SEQ. ID.No.23)
N1-9GGTCTCN
(SEQ. Identification No. 24) < SEP> N1-9CGAGA
(SEQ. Identification No. 25)
< tb> BbsI/Acc36I
(bipartite) < SEP> N1-9GAAGACNN
(SEQ. Identification No. 26)
TTCN1-9
(SEQ. Identification No. 27) < SEP> CAGGTN1-9
(SEQ. Identification No. 28)
N'1-9ACCTGCNNNN
(SEQ. Identification No. 29) < SEP> NNNNNNGTC
(SEQ. Identification No. 30) NNNNN'4 G
(SEQ. Identification No. 31)
< tb> < /TABLE>

Means: N any of the nucleotides A, G, C or T; N' that in each case to N at the corresponding position in the Gegenstrang complementary nucleotide X any nukleotidisches or nichtnukleotidisches element (if necessary. with an appropriate modification), which is qualified for a Kettenbildung.

[0045] The subscript numbers indicate the number of respective elements.
EMI27.1

EMI28.1

Means: N everyone of the nucleotides A, G, C or T; And the interface in the ?upper? strand, i.e. 5 ' - > 3 ' from left to right _die interface in the ?lower? strand, d.h.5'-> 3 ' from right to left

[0046] Preferential mating first and second restriction enzyme way of a DNA preferential by the type a IIS to tack of the use of the two classes of Oligonukleotiden and the left molecule for the synthesis of a nucleic acid, is the following: Eco31I/Esp3I (37 DEG C), BsaI/BsmBI (50 DEG C), BsmBI/BsaI (55 DEG C), BbsI/BspMI (37 DEG C), BspMI/BbsI (37 DEG C) BsrDI/BtsI (65 DEG C), BtsI/BsrDI (37 DEG C), BciVI/Bmrl (37 DEG C), AarI/AceIII (37 DEG C), EciI/BseRI (37 DEG C) and BmrI/BciVI (37 DEG C). (The temperatures indicated in staples are the Inkubationstemperaturen used with the respective mating.). The Isoschizomeren to these enzymes (BsaI: Bso31, Eco31I; BsmB1: Esp3I; BbsI: Bpi1, BpuAI; BspMI: Acc36I; BsrDI: Bse3DI, BseMI; BmrI: BfiI) represent potenzielle alternate candidates; partially. these are überexprimiert from klonierten vectors and in higher yield and/or. Purity produces. Isoschizomere are preferentially used also if the shelf-life of an enzyme is limited compared to its Isoschizomeren.

[0047] Now for example if BsaI is used as restriction enzyme, an overhang from four nucleotides is produced, which any sequence can exhibit. Since at each of the four nucleotide positions in principle all four nucleotides (A, G, C, T) can stand, each sequence consisting of four nucleotides can be represented with altogether 256 Linkern. A such left one can be hybridized then with a Oligonukleotid due to the Komplementarität of the sequences by part A of the Linkers with the einzelsträngigen part of the Oligonukleotides. If the overhang produced by the restriction enzyme amounts to two nucleotides, the appropriate left library will cover 16 elements, with an overhang of three nucleotides of 64 elements, with an overhang of five nucleotides 1024, with an overhang of six nucleotides of 4096 and with an overhang of seven nucleotides 16384 elements.

[0048] With such libraries that the part B exhibits any sequence with view of the individual Linkers apparently, however in the whole those is to be marked to left ones of an appropriate library the entire sequence area defined by the length of the overhang takes off and in each case a defined, D. h. do not cover randomized succession of nucleotides.

[0049] The managing conception of the generation of pairs from a Oligonukleotid of the first class, herein also as the first Oligonukleotid designated, and an appropriate left one, defined by a specific restriction enzyme, whereby the class of the Oligonukleotids is defining restriction enzyme the same, like the class of the Linkers defining restriction enzyme of the type IIS, can be accomplished now in same way for a pair from left one and Oligonukleotid of a second class, which by another, second restriction enzyme by the type IIS are defined, and then complexes are made of left one and Oligomer. After in this case another restriction enzyme is used by the type IIS, part A of the Linkers will differ from that

managing in connection with the first restriction enzyme from the type IIS described left one. Part B will be out-arranged however, again as a function of the length by the restriction enzyme by the type IIS produced end and altogether an appropriate sequence area will define.

[0050] By that managing described procedure are thus present typical way two left libraries, under specificity and/or. Komplementarität of the recognition place of the respective restriction enzyme of the type IIS with in each case a corresponding Oligonukleotid to hybridize know. After hybridizing and if necessary. Ligierung of the Linkers with the Oligonukleotid is phosphoryliert this typical way and the overhang by means of a polymerase, generated by part B of the Linkers, is filled up, so that the complex from Oligonukleotid and left ones is present now as glattendiges Oligonukleotid. This event is repeated for the Oligonukleotid of the second class and the appropriate left one (the second class). Subsequently, the two are ligiert with one another smooth-ended Oligonukleotide elongated around that left ones and split afterwards with one of the two restriction enzymes by the type IIS. Consequently it comes to an extension or the other Oligonukleotids. The number of added nucleotides is determined thereby by the length of the overhang, which of in each case the enzyme used for the cracking of the two ligierten smooth-ended Oligonukleotide is produced.

[0051] The purposeful structure of a defined nucleic acid will contain as part B the sequence, which is to be added to or a nucleic acid which can be developed already existing by use and repetition of the schematic reaction happening described above thereby possible that from the left library those left ones are selected. After cracking of the Ligationsproduktes from the two smooth-ended Oligonukleotiden one receives a abgespaltenes Oligonukleotid, which in the gene synthesis after the Sloning method, how it subject-matter of the international patent application WHERE 00/75368 is, be used can. In this method larger genes are manufactured by the fact that first in parallel reaction beginnings partial fragments are developed by sequenzielle Ligationen by unmodified doppelsträngigen Oligonukleotiden (so-called Splinkern) to a Oligonukleotide immobilizable over a modification (so-called Anchor). The Ligationsprodukte developing thereby is split after each step with the restriction enzyme, whose recognition sequence is contained in the anligierten Splinkermolekülen. Thus only in each case the variable part of the Splinker at the Anchor molecule remains, while the constant part is separated by the restriction enzyme and removed by a wash step from the reaction beginning. Depending upon the partial sequence which can be synthesized for each single step the Splinker from a library of all Splinker, for this needed, one selects. Subsequently, one half of in such a way received fragments is treated with the Anchor spezfischen restriction enzyme, the other half of the fragments with that Splinker specific enzyme. Each of these fragments exhibits now a einzelsträngigen overhang, which complementary is to the overhang of the fragment next in the succession of the gene sequence which can be synthesized. By Ligation of the fragments lying next to each other (so-called Transposition) the length of the now available fragments doubles itself, while the number of fragments halves itself. With each further Transposition the length of the partial fragments to only a fragment doubles itself is remaining finally, which contains under normal conditions the entire gene sequence which can be synthesized.

[0052] In its public formulated, this method, which is called herein also general the Sloning method, covers thus the following steps:

a) Make a Oligonukleotids, that available is manufactured by the following steps:

aa) coupling of a Oligonukleotids with an end to a solid matrix, whereby the coupling is made by a modification, and the Oligonukleotid a recognition sequence for a TypIIS restriction enzyme contains, which cuts outside of its recognition sequence,
off) addition of a further, at least partial doppelsträngigen Oligonukleotids with another recognition sequence for a TypIIS restriction enzyme, which cuts outside of its recognition sequence, than in step aa), whereby this Oligonukleotid cannot bind to the matrix,
AC) Ligation of the Oligonukleotide from step aa) and off) in orientation specified by the blocking that not to ligierenden ends,
ad) removing not spent reactant as well as enzymes,
ae) cracking of the Ligationsprodukts from step AC) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the nucleic acid sequence of the Oligonukleotids from step off) takes place,
af) separating of the reaction mixture of in step ae) received elongated Oligonukleotid from step aa),
AG) at least unique repeating of the steps off) to af),

b) Make a further Oligonukleotids, that available is manufactured by the steps:

ba) coupling of a Oligonukleotids with an end to a solid matrix, whereby the coupling is made by a modification, and the Oligonukleotid a recognition sequence for a TypIIS restriction enzyme contains, which cuts outside of its recognition sequence,
bb) addition of a further, at least partial doppelsträngigen Oligonukleotids with another recognition sequence for a TypIIS restriction enzyme, which cuts outside of its recognition sequence, than in step ba), whereby this Oligonukleotid cannot bind to the matrix,
UC) Ligation of the Oligonukleotide from step ba) and bb) in orientation specified by the blocking that not to ligierenden ends,
bd) removing not spent reactant as well as enzymes,
) cracking of the Ligationsproduktes from step UC) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the Oligonukleotid in step bb) takes place,
BF) Separating of the so elongated nucleic acid molecule of the reaction mixture,
bg) at least unique repeating of the steps bb) to BF), whereby following the last Ligation in step UC) and removing not spent reactant as well as enzymes the Ligationspordukt with a TypIIS restriction enzyme is cut,
whereby the cracking in Oligonukleotid in step ba) takes place,
c) Ligation of the Oligonukleotide from step A) and b) in orientation specified by the blocking that not to ligierenden ends,
d) Do not remove spent reactant as well as enzymes,

e) Cracking of the Ligationsproduktes from step C) with a TypIIS restriction enzyme, which cuts outside of its recognition sequence, whereby the cracking in the Oligonukleotid from step A) or b takes place),
f) Separating of the so elongated nucleic acid molecule of the reaction mixture.

[0053] The term of the solid matrix, how it is used herein, designates general any surface, at which a coupling can take place at least one of the reactants. In particular for this surface forms count such as filter, films, diaphragms, chip, sheets, ball (English. beads) and columns. These surface forms can be made of one of the following materials: Polymers such as plastics, for example polystyrene, polyacetate, polyacrylamides, polyvinylidene fluoride, agar eye, Sepharose, cellulose; Silicon, glass (silica glass) and silicagel. These materials can in or several the person skilled in the art admitted kind and ways modified to become.

[0054] The coupling can take place on sides of the Oligonukleotide via it, a modification internally, i.e. at a non-terminal nucleotide of the Polynukleotids, or terminal, i.e. at a terminal Nukloetid, is present. The latter will be possible in particular if the Oligonukleotid as a two-piece (English. bipartite) structure is present. Such modifications, which permit a coupling of a Oligonukleotids to a surface, in particular a modified surface, are to the specialists on the field admitted and cover for example biotin, Iminobiotin, Digoxygenin, Sulfhydrylgruppen, Diclycohexylcarbodiimid, Fluorescein, Acridin and Rhodamin.

[0055] The coupling can take place on sides of the solid matrix via or several of the following modifications: Avidine, like Streptavidin, monomeres Avidin, at the Tyrosinresten modified Avidin; Antibodies, in particular such, which are directed against those connections managing specified, and Sulfhydrylgruppen.

[0056] It is in the context of the abilities of the specialists on the field to determine suitable combinations of the managing modifications of the reaction partners.

[0057] In addition, in an advancement of the method after WHERE 00/75368 several gene variants simultaneous can be manufactured. In order to be able to manufacture partial fragments with terminals different sequences, for it the following modification of minutes is necessary: After the Ligation of a modified Splinkers to an elongated Anchor (after previous blocking of the existing connection places at the solid phase) the developed Anchor Splinker Ligationsprodukt is cut not with the Splinker specific Restriktionsendonuklease, but with that Anchor specific restriction enzyme. Thereby doppelsträngige DNA of molecules, which exhibit a einzelsträngigen overhang at an end, does not develop for no more however despite their modification is bound to the solid matrix. This DNA of molecules can be divided therefore now on different reaction containers and bound to a solid phase. After renewed blocking of free connection places, Ligation of new Anchormoleküle as well as cracking with the Splinker specific Restriktionsendonuklease can be anligiert now different Splinker ligiert, which differ in the overhang sequence, not however in the nucleotides bordering directly on the overhang, which form the next overhang after the following restriction. In this way several different partial fragments can be developed. These fragments are ligiert in the next step with Splinkern, which exhibit the same sequence following the overhang sequences again, so that all variants of fragments can be linked after the following cracking with the fragment following in the gene sequence which can be developed. In order to achieve an approximately äquimolare allocation of these different fragments, also all other, parallel manufactured Ligationsprodukte must be accordingly treated and portioniert. For this it is necessary that those fragments, which after the original method at the Anchor remain after that to cutting edges with the Anchor specific Restriktionsendonuklease only again with a Anchormolekül be ligiert must, in order to be able to continue the reaction. This aspect of the invention becomes in the Figs. 9 to 14 illustrates.

[0058] As required a polymerase nuclear chain reaction (PCR) can follow, in which as primer Oligonukleotide are used, those to the constant parts of the Anchor and/or after the last Transposition. Splinker are more complementary. In this way losses can become by the allocation of the reactions again compensated. Preferably here thermalstable polymerases with Proofreading function are used, in order to introduce if possible few additional errors.

[0059] A similar method can be used, in order to prevent that shortened Ligationsprodukte, which can result from inkomplette cracking with the Splinker specific Restriktionsendonuklease is kidnapped not into the following Transpositionsreaktionen (= transmission from elongated Splinkermolekülen to that cutting edges with the Anchor specific Restriktionsendonuklease) and to Deletionsmutationen to lead in such a way to be able. Here in place of the last in each case Splinkermoleküle at the end of the Ligation/restriction cycle Anchormoleküle are ligiert, which contain however a recognition sequence for the Splinker specific restriction enzyme (so-called Splinker Anchor). It is ensured by a blocking of still existing free connection places of the solid phase that the Splinker Anchor is coupled only by means of Ligation to the elongated Anchor to the solid phase. After the cracking of the Ligationsprodukte with that, developed in such a way, only those molecules, which received a modified Splinker Anchor, point Anchor specific restriction enzyme, which knows appropriate modification up and therefore in a next step after transferring into a new reaction container by connection to a solid phase from not modified partial fragments is separated in the last step. This aspect of the invention becomes in the Figs. 15 to 17 herein continues to describe.

[0060] The moreover one also the reversal of the 00/75368 procedure described in the application WHERE is possible: instead of developing the gene fragments at the Anchor Oligonukleotide coupled to a solid phase, the Ligationen can take place also in principle in solution, whereby modified in each case Splinker Anchor is ligiert instead of Splinker Oligonukleotide. After the restriction with the Splinker specific Restriktionsendonuklease remain now the not cut Ligationsprodukte at the solid phase during the fragments freely set by the restriction into the further reaction beginnings to be transferred can. Preferential way can with this proceeding enzymes be used, which even a modification are thus likewise held back to carry and in the reaction beginnings. A heat denaturing of the enzymes is no longer necessary thereby. This aspect of the invention becomes in the Figs. 18 to 20 in the detail described.

[0061] In principle up-synthesized partial fragments can be transferred by combination in the last both paragraphs described methods as desired between Anchor and Splinker Anchor back and forth (transfer reaction). An identical overhang sequence presupposed, so arbitrary gene fragments can become from different syntheses combined with one another. This ?mini Exon Shuffling? is suitable for example outstanding for the manufacturing of designer proteins or for the optimization of enzymatic properties by the combination of mutants with increased activity and/or. Stability.

[0062] In the application WHERE the 00/75368 described gene synthesis procedure was in the selection of nucleic acids which can be synthesized to that extent reduced the recognition sequences of the used Anchor and Splinker specific Restriktionsendonukleasen in the sequence which can be synthesized was not allowed to arise, since this would lead to internal cracking of the partial fragments. This restriction could be gone around by the fact that when building such a sequence up a Splinker with a recognition sequence for an alternative Restriktionsendonuklease is used and the Ligationsprodukt then afterwards with the appropriate Methylase is treated. The methylierten internal sequences are then protected against the cracking with the appropriate Restriktionsendonuklease, while the Anchor and Splinker can still be separated. This aspect of the invention becomes in the Figs. 21 to 22 further implemented.

[0063] Finally an improvement of the method of the original application can be achieved by the fact that the inset of Splinker Oligonukleotiden with one is possible for only three Nuleotide large overhang, although so far no pair of Restriktionsendonukleasen admits is, which produce three nucleotides a long overhang and exhibit from each other distinguishable recognition sequences beyond that. The problem that the gene fragments cannot be set for the other end in the Transpositionen on then by cracking either or together, can be gone around by the fact that the last Splinker which can be added is an adapter, i.e. a long supernatant end exhibits three nucleotides, but a recognition sequence for a restriction enzyme contains, which produces four nucleotides a long overhang. The inset of such Splinker adapters is of advantage to that extent, only a Splinkerbibliothek of smaller complexity is necessary (4096 instead of 65536) and three nucleotides long overhangs to be more complementary never can. Since besides the genes can be developed then in the triplet raster, a further restriction of the complexity is possible for coding regions, since for all Codons Splinker ready do not have to be provided. Altogether 256 different Splinker adapters are needed, in order to be able to cover all sequence variants; if one is limited to the 30 most frequent Codons, 120 would be sufficient. This aspect of the invention becomes in the Figs. 2 to 8 more near describes.

[0064] The main aspect of the present invention is based on the fact that it is possible with the help of the methods described here to reduce the value of the Oligonukleotid library necessary for the syntheses of arbitrary genes by a combinatorial making from two smaller libraries crucially. From this proceeding further clear advantages result, no more standardized elements with lengths by 30 to 40 nucleotides to nevertheless are used, but rather left molecules opposite the already very favourable Sloning method, which lengths of typical way 6 to 11 nucleotides exhibit. Thereby the attainable Aufreinigungsgrad of the standardized Oligonukleotidbausteine is extremely improved and thus the basis for a very reliable and automizable nucleic acid synthesis is created.

[0065] A further advantage of the method according to invention, as it results from the managing display, consists of the fact that the value of the library amounts to that left ones in case of an overhang of four nucleotides only 256, whereby this must be manufactured for each restriction enzyme by the type IIs, which is used on sides of the Oligonukleotide, accordingly. Thus that altogether in the example managing specified 512 different left ones as well as two Oligonukleotide (in each case one for each class) must be only manufactured, opposite altogether 65,536 Oligonukleotiden result (with the use of restriction enzymes, which produce an overhang in relation to their recognition sequence of four nucleotides).

[0066] A completely particularly preferred further embodiment both regarding that left one, as well as itself the libraries resulting in from it can be realized if as in case of the pair of restriction enzymes Eco31I/Esp3I, the recognition sequence differs only in a nucleotide. Under these circumstances it is possible that instead of necessary 512 (256 left ones for class 1 and 256 left ones for class 2) different elements only once 256 different left ones must be manufactured, if, in that the two restriction enzymes of the pair of restriction enzymes do not differ the nucleotide in part A of the Linkers, but in the terminal part of the doppelsträngigen range of the Oligonukleotides is arranged.

[0067] In the following table 2 sequences for representatives of the two classes are indicated by Oligonukleotiden as well as the restriction enzyme of the type IIS, corresponding to them, whose recognition sequence either completely or partly in that 3 ' - OH-overhang is present.
EMI38.1

[0068] The nucleic acid molecule libraries revealed herein consist of a majority of the einzelsträngigen nucleic acid molecules according to invention, as they are revealed herein. The term einzelsträngiges nucleic acid molecule and left one become herein, if differently indicated, synonymously does not use. Preferential way cover these nucleic acid molecule libraries the entire sequence area, as it is defined by the length of the overhang (part B of the Linkers). It is however also to the extent of the present invention that only a part of the appropriate left ones and thus a part of the sequence area are contained in the nucleic acid molecule library. Beyond that the relative relationship of the individual molecules can be in a such library to each other both directly and differently trained. For example it is in the context of the present invention that such sequences, which occur comparatively rarely in the sequences which can be synthesized or natural sequences are accordingly underrepresented compared to other, more frequently occurring sequences.

[0069] As was already revealed on the basis the managing description, both the einzelsträngigen nucleic acid molecules, D can. h. Left, and the nucleic acid molecule libraries in the context of a method for the preparation of a nucleic acid molecule to be used. Preferential way acts it with this method around a method with sequenzieller Ligation of Oligonukleotiden in sequence-independent way, as it is exemplarily described in the international patent application WHERE 00/75368. Those herein as Oligonukleotide of the first class defined Oligonukleotide, whereby the allocation effected via it that a Oligonukleotid of the first class a recognition sequence first restriction enzyme of the type of a IIS or a part of it or a sequence complementary in addition enclosure and a Oligonukleotid of the second class covers a recognition sequence second restriction enzyme of the type IIS or a portion of, it different of the first restriction enzyme, or a sequence complementary in addition, thereby a so-called ?Anchor? - Oligonukleotid can be, i.e. a modification carry, which permits an immobilization of the Oligonukleotids at a solid phase, and which other class a so-called ?Splinker? - Oligonukleotid, which exhibits one directly or different one fissile modification. Otherwise ?Anchor? - and ?Splinker? - correspond to Oligonukleotide the structure from a einzelsträngigen and a doppelsträngigen range, described herein for the Oligonukleotide, as well as optionally a loop.

[0070] The method according to invention plans with the fact that a Oligonukleotid of one by the presence of the recognition sequence, whose complementary sequence or in each case a part of it defined, a first restriction enzyme of

the type IIS class 1 is made available, whereby this Oligonukleotid can being trained regarding the recognition sequence for the first restriction enzyme like above revealed. This in the following as the first Oligonukleotid designated Oligonukleotid can exhibit a modification, which permits a fixing or an immobilization of the first Oligonukleotids at a surface, to preferential way at a solid matrix. Preferential way is in such a way trained this modification that a splitting off of the Oligonukleotids bound to the surface can take place. To this first Oligonukleotid a left one according to invention under suitable conditions is in addition-given, so that a hybridizing between the first Oligonukleotid and that takes place left ones. Hybridizing is based on the Komplementarität of part A of the Linkers with the einzelsträngigen part of the Oligonukleotids. A doubling rank is formed by hybridizing, which contains the complete recognition sequence of the mentioned restriction enzyme of the type IIS. The proportions between the first Oligonukleotid and that left ones are out-arranged according to the requirements of an efficient Ligation, whereby typical way it is intended that the comparatively smaller left ones in the surplus to the first Oligonukleotid is in addition-given.

[0071] As next step can after treatment with a Kinase of the 5 ' - over-hanging part of the first Oligonukleotides filled up and thus smooth-end to be made. Preferential way becomes before filling up, typical way using the Klenow fragment of the T4 DNA polymerase, for which surplus of the Linkers removes. This distance can take place in the case that the first Oligonukleotid at a solid matrix is immobilized, via appropriate wash steps. Alternatively it can be intended that an isolating of the different molecules, in particular a separation of the Linkers if the first Oligonukleotid not immobilized is by means of suitable separation techniques, as for example gel electrophoresis or gel filtration, to take place. Apart from the preferential way taking place distance of the ligierten surplus of Linkern either simultaneous become or in different steps also the further components of the reaction beginning, D. h. Kinase, Klenow fragment and the nucleoside triphosphates not converted with the replenishment remove.

[0072] Parallel in addition or in the follow-up in addition afterwards a Oligonukleotid of the second class, herein also second Oligonukleotid, is called made available, whereby itself this Oligonukleotid and/or. the class thereby distinguishes that it completely or partly the recognition sequence of a restriction enzyme of the type IIS, different of the class 1, and/or. the complementary sequence is converted for this to cover and these now with an appropriate left one, whose part is more complementary A to the einzelsträngigen range of the Oligonukleotids, and after Ligation and filling up at the end a glattendiges Oligonukleotid also here is present. Also the second Oligonukleotid can be present, now provided with that left ones and accordingly filled up, at a surface immobilized.

[0073] In a next step the smooth-ended Oligonukleotide is brought with one another in contact, whereby preferential way either the first Oligonukleotid or the second Oligonukleotid at a surface immobilized is present. It is however also in the context of the method according to invention that both smooth-ended Oligonukleotide in solution are present. The two smooth-ended Oligonukleotide, which opposite the respective Ausgangsoligonukleotiden in each case around the length of the left overhang at their 5 ' - end elongated are, using a Ligaseaktivität are ligiert. The not converted molecules as well as the used enzymes can do after that the specialists on the field admitted methods easily to be removed, so for example if the entire reaction in solution took place, by gel electrophoresis, if one is present the Oligonukleotide at a surface immobilized and thus also the Ligationsprodukt, by washing using suitable washing solutions.

[0074] In a further step using one of the two restriction enzymes from the type IIS a Oligonukleotid is separated from the Ligationsprodukt between the two smooth-ended, filled up Ausgangsoligonukleotiden. This differs opposite the originally used Oligonukleotid by the fact that this contains the variable nucleotides (part B) of the Linkers ligierten before, just as the variable nucleotides of the second Linkers which was connected with the second Oligonukleotid.

[0075] With the embodiment of the method according to invention, with which at least one is the Oligonukleotide to a solid phase immobilized, still the step follows before the cut step by the restriction enzyme of the type IIS, which from the smooth-ended and filled up first Oligonukleotid and smooth-ended and filled up second Oligonukleotid developed Ligationsprodukt is separated from the surface by cracking of the connection between the Ligationsprodukt and the solid surface, trained by the modification existing in the Oligonukleotid.

[0076] The kit according to invention covers at least one of the einzelsträngigen nucleic acid molecules according to invention, D. h. Left. Preferential way covers a such kit one of the nucleic acid libraries according to invention, or a part of it. In an embodiment the kit covers beyond that still suitable buffers, enzyme activities such as ligases, Topoisomerasen, 3 ' - 5 ' - Exonukleasen, Phosphatasen, type IIS Restriktionsendonukleasen, or suitable surfaces. Preferential way covers the kit two different restriction enzymes of the type IIS, which produces overhangs of the same length for preferential way. It can be intended that the surfaces already exhibit or several the standardized Oligonukleotide.

A such kit serves typical way for the preparation of a nucleic acid

[0077] The term nucleic acid covers herein preferential way Desoxyribonukleinsäure.

[0078] The present invention is continued to describe now on the basis the following figures and examples, resulted from those further features, embodiments and advantages of the invention. Shows and/or. show

Fig. 1 the expiration of the method according to invention;
Figs. 2 and 3 the production of a library of Splinker molecules with an overhang of three nucleotides;
Figs. 4 and 5 a method for the structure of a library, which the transition of Splinker and/or. To Anchor molecules with three nucleotides a long overhang to such with four nucleotides a long overhang permit;
Figs. 6 to 8 an embodiment of the Sloning method using Anchor and Splinker molecules with three nucleotides overhang is enough;
Figs. 9 to 14 the substantial steps of the method according to invention to the simultaneous making of different gene variants under use of the Sloning method;
Figs. 15 to 17 the different steps with the distance of not split false sequences;
Figs. 18 to 20 the different steps with the gene synthesis in solution, with which it concerns a further embodiment of the SloningVerfahrens;
Figs. 21 and 22 the substantial steps with the synthesis of DNAFragmenten with internal Methylierung according to the

present invention; and
Fig. 23 the method (between) - of product amplification, as it can be accomplished in the framework by different steps of the Sloning method.

[0079] Fig. 1 shows the expiration of the method according to invention, with which for the reduction of the value of the library from Anchor and Splinker molecules a einzelsträngiges left molecule is used. In a first step a Oligonukleotid 1 is made available, also as generic Splinker or master Splinker designated are covered and a einzelsträngigen range of comprising five nucleotides as well as a doppelsträngigen range of comprising seven nucleotides as well as a loop consisting of four nucleotides. The loop carries a modification X, which is suitable to bind Oligonukleotid 1 to a solid surface. Preferential way concerns it thereby a reversible connection. The Oligonukleotid 1 exhibits a supernatant 3 ' - OH-end. 5 ' - end is phosphoryliert chemically or enzymatically.

[0080] In a second step Oligonukleotid 2 is made available, which is called herein generic Anchor or masters anchor. Also Oligonukleotid 2 consists comprising five nucleotides, a doppelsträngigen range of comprising six nucleotides as well as a loop of comprising four nucleotides of a einzelsträngigen range. The loop carries a Biotinylierung, which permits a connection of the Oligonukleotides to 2 to a surface. Similarly as Oligonukleotid 1 that stands for 3 ' - OH-end around five nucleotides over and 5 ' - end is chemically or enzymatically phosphoryliert. The supernatant 3 ' - OH-end of Oligonukleotid 1 represents thereby the recognition sequence of restriction enzyme X.

[0081] Both Oligonukleotid 1 and Oligonukleotid 2 are bound independently to a solid carrier, in the case of biotinylierten Oligonukleotiden at Streptavidin coated Beads or micro titer plates after the manufacturer data. To the immobilized Oligonukleotiden 1 and 2 a left one is then in addition-given in each case. In the available case part A of the Linkers covers the sequence CGAGA and corresponds thereby to the complementary strand of the last 4 nucleotides the type IIs of restriction enzymes Eco31I and Esp3I and hybridizes with the appropriate single strand of the Oligonukleotids 1. Subsequently, a Ligation takes place using a league SE activity and it comes to the construction of the complete recognition sequence of restriction enzyme Eco31I. Same takes place at the Oligonukleotid 2 likewise immobilized at a surface. After Ligation of the Linkers at Oligonukleotid 1 and/or. Oligonukleotid 2 gets over part B of the Linkers in both felling and defines the range and/or. the nucleic acid sequence, that and/or. in the context of the synthesis to be developed is. This supernatant end is filled up specifically after Kinasebehandlung and Klenow Polymerasebehandlung by means of appropriate nucleoside triphosphates, so that at the end a filled up Oligonukleotid (1) and/or. Oligonukleotid (2) at the surface immobilized is. As a function of the nucleic acid which can be synthesized thereby those left ones are selected whose part B exhibits the desired sequence. In Fig. 1 represented methods was ensured by suitable choice of the pair of restriction enzymes that both for extending and/or. Fill up by Oligonukleotid 1 and by Oligonukleotid 2 the same left library to be used can. This became possible thereby that the sequence of the Oligonukleotides 1 and/or. Oligonukleotides 2 at the transition between the Oligonukleotid and that was out-arranged in such a way left ones that after Ligation the recognition sequences different for the two restriction enzymes were trained.

[0082] As next step the distance can take place from Oligonukleotid 1 and/or Oligonukleotid 2 from the surface. Subsequently, it comes to the Ligation of the two filled up Oligonukleotide 1 and 2. In a further reaction step then the Ligationsprodukt with one of the two restriction enzymes is split by the type IIS, in the available case with Esp3I. In this way complete Splinkeroligonukleotide with all 65536 possible Oktamerendsequenzen can be produced.

[0083] The figures 2 and 3 show the production of a library of Splinker molecules with an overhang of three nucleotides. As herein revealed and from that aspects managing specified obviously, for example with the production way Anchor and Splinker molecules used with an overhang of three nucleotides, preferential by gene variants and in particular of gene variants, which concern coding nucleic acids. To that extent it is an aspect of the invention to make a method available to the production of a library of Splinker molecules and/or. Anchor molecules with an overhang of three nucleotides. Exemplarily for the production of such molecules the production of a library with Splinker molecules with an overhang of three nucleotides is represented in the figures 2 and 3. In connection with the description of the figures thereby the term of the Anchors or Anchor molecule designates 2 and 3 in accordance with Oligonukleotid aa) and/or. ba) the Sloning Verfahres and/or. the term of the Splinkers or Splinker molecule in accordance with Oligonukleotid off) and/or. bb) the Sloning method.

[0084] The structure of the library begins thereby on the basis of a Anchor molecule which a modification carries, which permits a coupling to a solid matrix, as well as a Splinker molecule, which likewise carries a modification, which permits a coupling to a solid matrix, whereby this Splinker modification of preferential way is fissile. To a Anchor molecule, more exactly the generic Anchormolekül (Splinker which can be synthesized are and/or for all. Anchor Oligonukleotide equal) is called, in addition-given a einzelsträngiges nucleic acid molecule, herein also left one, which consists in the available case of a Nonamer, a comprising part B and a portion A. The part A is thereby complementary to that 5 ' - overhang of the Anchor molecule. The part B covers three nucleotides, which cover any sequence. In the same way a more preferentially way-generic Splinker molecule a einzelsträngiges nucleic acid molecule one proceeds, i.e. a left one, is admitted which likewise consists of a part A and part B, whereby the parts A and B are just as trained in principle as in case of the Linkers in addition-given to the Anchor molecule. With view of the fact that at each of the three, in Fig. to stand by means of 64 different einzelsträngiger left ones the entire sequence area can do, can 16 (A) with N designated positions one of the four nucleotides, i.e. all possible molecules, which can differ in these three nucleotide positions, to be illustrated. Preferential way is the length of the part A of the Linkers six nucleotides, whereby however also left one with larger and smaller lengths is contained in the scope of protection by part A.

[0085] By hybridisation of the Anchor molecule and/or. the Splinker molecule with in each case one that altogether 64 Linkern, then altogether 64 different Anchor molecules know and/or. Splinker molecules to be produced, which differ in each case in the part B. Part A of the Linkers is more complementary thereby typical way to the recognition sequence, or a portion of it, Anchor and/or. the Splinker specific restriction enzyme or the sequence complementary in addition. The over-hanging ends of the Anchor filled up with that left ones and/or. Splinker molecules by a polymerase, for example by Klenow polymerase is then filled up and produced thus Anchor molecules and Splinker molecules with smooth ends.

[0086] Preferential way is coupled the Anchor molecule just like the Splinker molecule to a surface or a solid matrix. The modification of the Splinker molecule can be split preferential way under mild conditions, so that the filled up Splinker

can be solved from the surface and be added a Ligationsansatz with a suitable smooth-ended Anchor molecule.

[0087] In a next step the Ligationsprodukt from step (C) with a Anchor specific Restriktionsendonuklease is then split by the type IIS, whereby Splinker molecules with a long overhang originate in to three nucleotide. In same way the cracking can take place in principle by means of Splinker specific Restriktionsnukleasen. By this process can be produced on the basis of altogether 64 different einzelsträngigen Linkern, which in three successive nucleotides differ, altogether 4,096 different doppelsträngige Splinker molecules, which can be used then as output library for a Sloning method in the described procedure.

[0088] With the structure according to invention of gene fragments from Anchor and Splinkermolekülen with an overhang of three nucleotides it is necessary, Anchor molecules and/or. Splinker molecules to make available, which were appropriate for three nucleotide overhang exhibit, however the recognition sequence for a restriction enzyme of the type IIS carry, which produces overhangs with a length of in, for two, four, five or more nucleotides. This is necessary, because for the lead-through of the Sloning method always at least two restriction enzymes must be used by the type IIS with distinguishable recognition sequences, which produce overhangs of the same length beyond that. At this time are however at restriction enzymes of the type IIS, which produce three nucleotide a long overhang, only Isoschizomere admit, which recognize the same sequences. . One of these restriction enzymes is SapI. Before the transition to the Transpositionsphase (i.e. the linkage of the parallel synthesized partial fragments, which must be cut in pairs in each case with different restriction enzymes) it is thus first necessary with use of a library of Splinkern with 3 nucleotides a long overhang, fragments with a equivalent long overhang (e.g. to create consisting of 4 nucleotides).

[0089] The Figs. 4 and 5 points a method to the structure of a library, which the transition of Splinker and/or. To Anchor molecules with three nucleotides a long overhang to such with four nucleotides a long overhang permit. In principle similarly one proceeds, as with that managing described methods for the structure of a library from Splinker and/or. For Anchor molecules with a nucleotide overhang is enough for three. The substantial difference exists in the arrangement of the left molecule, which, as in (A) represented, exhibits a length of two nucleotides in the available case. To that extent 32 einzelsträngige left molecules are sufficient, 16 Anchor specific as well as 16 Splinker specific, around a complete library, i.e. a library, which contains all possible sequences, with which itself the two last 5 ' - terminal positions differentiate, to construct. In step (D) with the Anchor specific Restriktionsendonuklease is then cut, in the available case SapI, which produces three nucleotide a long overhang. With this method then those can be produced altogether 256 different doppelsträngigen molecules, which permit the transition of Splinker and Anchor molecules with three nucleotides a long overhang with their use to such with four nucleotides a long overhang and therefore also Splinker Adaptoren are called.

[0090] On the basis of the two managing described libraries and the methods to their preparation then a structure can take place from gene fragments from Anchor and Splinker molecules with three nucleotides a long overhang. The appropriate method is represented in the figures 6 to 8. In connection with the description of the figures the term of the Anchors or Anchor molecule designates in accordance with Oligonukleotid aa) and/or. ba) the Sloning Verfahres and/or. the term of the Splinkers or Splinker molecule in accordance with Oligonukleotid off) and/or. bb) the Sloning method.

[0091] If one goes from the sequence which can be synthesized explained in (A) (called herein also goal sequence), this can be arranged in the available case into the parts A and B. The part A consists just like the part B of 9 nucleotides, which are divided to three nucleotides in three groups each in each case. Accordingly the first tripartite group of the nucleotides can be transferred to the Anchor molecule on the basis of a Anchor coupled to a solid matrix by Ligation of a first Splinker molecule. By cracking of the Ligationsproduktes in step (B) by means of three nucleotides long overhangs the producing restriction enzyme of the type IIs (SapI) further three nucleotides a long overhang is produced, to which in the step (C) a second Splinker molecule is ligiert, which afterwards restriction enzyme Splinker specific by cracking with that is separated. In the same way with a third Splinker molecule is proceeded, whereby the Ligationsprodukt will then receive in accordance with (D). In step (E) is used after cracking of the Ligationsproduktes from (D) now an Splinker adapter, which permits the transition of an overhang from three nucleotides to an overhang from four nucleotides. Becomes after Ligation this herein also as adapter Splinkers or 3-> 4 - Adapter-left designated Splinker molecule with that Splinker specific restriction enzyme split, in the available case with Eco31I, now no more is produced three nucleotides a long overhang, but four nucleotides a long overhang and thus the conditions for a Transposition in the context of the Sloning method production, with which two restriction enzymes co-ordinated one on the other are used by the type IIS.

[0092] In the steps (B) to (E) of Fig. 7 one proceeds exactly the same in principle, as with the steps (B) to (E) of Fig. 6, whereby however the Splinker 3 nucleotide here a long overhang used in step (B) exhibits, which corresponds to the first triplet of part (B) of the goal sequence. After the third triplet was ligiert by part B by appropriate Splinker molecule to the elongated Anchor molecule, with SapI one cuts again and then a further Splinker molecule uses, which covers the first triplet of part C of the goal sequence. In order to create the condition for a Transposition also here, then with that Anchor specific restriction enzyme of the type IIS, in the available case with Esp3I, is cut and thus four nucleotides a long overhang is produced. The goal sequence from part A and part B, like in (A) of Fig. 8 represented, now by the fact it is produced that the fission product, D. h. the Anchor molecule with four nucleotides a long overhang from figure of 6 (F) with four nucleotides overhang is enough for exhibiting Splinker molecule from step (F) of Fig. 7 one ligiert.

[0093] The Figs. 9 to Fig. 14 points the substantial steps of the method according to invention to the simultaneous making of different gene variants under use of the Sloning method. In connection with this further embodiment of the Sloning method it is to be marked that here the substantial point is to be seen in the fact that a correctly elongated Anchor molecule or a correctly elongated Splinker molecule is divided on several reaction beginnings, preferential way in different reaction containers. This allocation is not unproblematic in particular if the modification of the Anchor and/or. Splinker molecules a coupling to a solid matrix permit, which do not permit a release of the mentioned molecules of the same, like for example with the use non-fissile modifications or modifications, those to a very stable reciprocal effect between nucleic acid and/or. Leads modification and solid matrix, is the case.

[0094] As into the Fig. 9 to Fig. 14 represented, is essentially this embodiment of the Sloning method by the specific arrangement of the steps aa) to AG) and/or. ba) to bg) characterized. In connection with the description of the figures

the term ?Anchor? or ?Anchor molecule? designates 9 to 14 in accordance with Oligonukleotid aa) and/or. ba) the Sloning Verfahres and the term ?Splinker? or ?Splinker molecule? in accordance with Oligonukleotid off) and/or. bb) the Sloning method.

[0095] If free connection places of the solid matrix were blocked, of a normal (not modified) Splinker Oligonukleotids a particular Splinker molecule can be ligiert to the elongated Anchor molecule in place. This particular Splinker molecule contains a modification, which permits a coupling to a solid matrix. (A). In a next step (B) takes place the cracking of the Ligationsproduktes with that, developed in such a way, Anchor specific restriction enzyme from the type IIS. In consequence of this cracking an elongated Splinker molecule is set free, which carries a modification and in the solution of the reaction beginning is contained. This reaction beginning, more exactly said the liquid projection of it, is then aliquotiert to the extent, how this is desired, in particular to the extent, as different gene variants are to be produced. For example three if gene variants are to be produced, how illustrates 9 to 14 in the figures herein exemplarily, an allocation of the liquid projection in altogether three aliquots takes place.

[0096] Each aliquot, which contains a separated, elongated Splinker molecule with a modification, is transferred into its own reaction container, whereby in consequence of the modification the elongated Splinker molecule is bound to the solid matrix (C). In Fig. 10 (C) with reaction 1, reaction 2 and reaction 3 designated illustrations represents those altogether three reaction beginnings described exemplarily herein.

[0097] To each reaction beginning a Anchor molecule is then in addition-given, which is hybridized in consequence of the Komplementarität of the over-hanging ends with the Splinker molecule coupled to the solid matrix and ligiert afterwards using suitable ligases. After the Anchor molecules themselves carry again a modification, which to a solid matrix it permits a coupling it is necessary that before addition of the Anchor molecules the connection places for this matrix are blocked, so that a coupling of in addition-given Anchor molecules is omitted to the solid matrix. This blocking step is then not necessary, if the modification of the Anchor molecules does not permit a coupling to the solid matrix in the reaction beginning, in which is contained to solid upper subject coupled Splinker molecule.

[0098] In einem nächsten Schritt wird sodann das Ligationsprodukt mit einer Splinker-spezifischen Restriktionsnuklease vom Typ IIS gespalten (E). After in step (D) in Fig. 10 the Anchor a modification carries, however to the solid matrix to couple cannot, after cracking of the Ligationsproduktes of the elongated Anchor in the liquid projection of the reaction beginning will be, whereas the Splinker molecule remains bound to the solid matrix in consequence of the coupling over the modification. Each of the projections is then transferred into a further reaction container, whereby the elongated Anchor contained in it is coupled in consequence of its modification to the surface (E).

[0099] To in such a way immobilized Anchor molecules to everyone of the appropriate reaction beginnings different Splinker molecules are in addition-given, whereby the Splinker molecules differ in a variable range, which itself that 5 ' - attaches over-hanging end. Preferential way exhibits itself the following variable range a length from 1 to 9 nucleotides, whereby 3 nucleotides are preferential, since thereby a Codon can be made available for a coding sequence specifically. In Fig. 11 (F) represented three reaction beginnings differ the Splinker at the positions 4 to 6 (AGA, CCG and/or. GTZ). Now in a next reaction beginning if the Ligationsprodukt from step (F) with a Splinker specific Restriktionsendonuklease, in such a way received, is split, differently elongated Anchor molecules develop in the reaction beginnings. In the reaction beginning 1 the variant covers then TTT, in the reaction beginning 2 GGC and in the reaction beginning 3 CAA (G). In a next step different left molecules, whose overhang is complementary to the overhang of the immobilized Anchor molecules, are then in addition-set. In consequence of the variability of the overhang of the individual Anchor molecule in the reaction beginning thus such Splinker molecules are to be in addition-admitted to each reaction beginning, which exhibit an accordingly complementary sequence, so that each reaction beginning requires another left ones (Fig. 7 (H)). Preferential way differ the different Splinker molecules only in this range. Subsequently, a further cracking of the Ligationsproduktes received in step (H) takes place by means of a Splinker specific Restriktionsnukleoase (J). Effected in a next step then a Ligation of a further Splinker molecule, that is more complementary to the respective overhang of the elongated Anchor molecule in each of the three reaction beginnings. Here the same left ones, be exhibited there the overhangs of the elongated Anchor molecule produced in step (J) an identical sequence can be used in each case. With the Splinker molecule added in step (J) it can concern thereby such, which makes the transition of one, as in the figures of 4 and 5 represented, three nucleotides for long overhang possible to four nucleotides a long overhang. This transition using the Oligonukleotids, which exhibits three nucleotides a long overhang, whose leads recognition places for a restriction enzyme of the type IIS with cutting edges of the Oligonukleotides with the same to four nucleotides a long overhang, herein also as Splinker adapter designated. In the described embodiment particular herein it is intended that this Splinker adapter exhibits a modification, which permits a coupling to the surface to a solid matrix. As a function of it, whether the Ligationsprodukt with a Splinker specific restriction enzyme, received in step (J), is split by the type IIS it will be received, the elongated Anchor molecules in the different beginnings, coupled represented in step (C), to a solid matrix, or, with use of a Anchor specific restriction enzyme from the IIS into (L) represented elongated Splinker molecules. In the first case the gene variants on the Anchor side, in the second case on the Splinker side are arranged. The arrangement of the Splinker molecule with a modification supplies the condition that in such a way elongated Splinker molecule can be used as Anchor molecule in a Transpositionsschritt of the Sloning method.

[0100] Although on the basis the figures 9 to 14 the unique introduction of a gene variant was only described, it is in the context of available revealing that this multiple can be made. Be enough for Splinker over not unnecessarily and/or. Anchor molecules too receive and thus the advantage of the parallel synthesis, i.e. Lead-through of Transpositionen to give up will take place less than five times less typical way as ten times, preferential way the installation of a gene variant, as managing described.

[0101] The Figs. 15 to 17 shows the different steps with the removing of not split false sequences according to the present invention. In connection with the description of the figures the term of the Anchors or Anchor molecule designates 1 to 3 in accordance with Oligonukleotid aa) and/or. ba) the Sloning Verfahres and the term of the Splinkers or Splinker molecule in accordance with Oligonukleotid off) and/or. bb) the Sloning method.

[0102] As is the case for the further embodiments altogether revealed herein than Sloning methods marked method to

the making of nucleic acid molecules by parallel synthesis, can the nucleic acid molecule, which by the steps aa) to AG) one manufactures, with the Oligonukleotid, which in accordance with the steps ba) to bg) one manufactures, with one another to be linked. This linkage is called herein also Transposition.

[0103] With the lead-through of the Sloning method the situation can occur that in the context of the sequenziellen addition of Splinker molecules from the library, which is called herein also structure synthesis incorrect intermediate products develop such that (for example by incomplete cutting edges for Splinker molecule specific type IIS of restriction enzyme) in the reaction beginning both correctly elongated, and incompletely elongated Anchor and/or. Splinker molecules are present. The presence of such incompletely elongated molecules would lead with their use as an element in the Transposition to an incorrect sequence. Of this reason the need consists to train the Sloning method in such a way that it is placed surely that only correct sequences, in particular on the level of the Anchor molecules, in the Transpositionphase, D. h. the parallel linkage of the developed gene fragments, to be used. In the context of the present invention this takes place via the fact that before the last Ligation of the Anchor molecule with a Splinker, i.e. before the Ligationsprodukt received from it is transponiert, a modified Splinker molecule is used, whereby the modification consists of the fact that the Splinker, in principle comparably, carries a modification, which permits a coupling to a solid matrix for the Anchor molecule. Since it is not possible, molecules of the reaction beginning, which were not split in the previous step with that Splinker specific restriction enzyme by the type IIS, a such modified Splinker molecule anzuligieren, are provided only the Ligationsprodukte of the correctly elongated Anchors with the last in each case (modified) Splinker molecule with a modification. The incompletely elongated Anchor molecules exhibit the Splinker molecules used therein which do not carry such modification as the product from a previous Ligation (see Fig. 1 (A)).

[0104] In a further step (B) takes place the cracking of the elongated Anchor molecules with that, existing in the reaction beginning, Anchor specific restriction enzyme from the type IIS. The fission products not coupled to the solid matrix are to correctly elongated Splinker molecules, the one marker carry as well as inkomplett elongated Splinker molecules, which do not carry tag. (B). The elongated Splinker molecules in such a way received become preferential way into a new reaction beginning and/or. new reaction container transfers. This vessel exhibits a surface as solid matrix, which permits a coupling by means of the modification existing at the correctly elongated Splinker molecule. Thus it comes to an immobilization of the correctly elongated Splinker molecules, whereas the not correctly elongated Splinker molecules, to which the modification permitting a coupling to the solid matrix is missing, bind not to the solid matrix. By one or more wash steps are removed the not correctly elongated Splinker molecules from the reaction beginning. Bound at the matrix the correctly elongated Splinker molecule, that remains to (C) with use from fissile modifications from this solid matrix to be again removed can and then with the originally used Anchor molecule be ligiert can whereby the sequence of the Anchormoleküls is identical with the sequence of the originally used Anchormoleküls.

[0105] Alternatively and if the modification does not permit a splitting off of the correctly elongated Splinker molecule, a Anchor molecule can be in addition-given to the reaction beginning, which contains only the immobilized, correctly elongated Splinker molecule, which carries a modification, which permits a coupling to a fixed matrix, however in this case at a surface is not bound. The connection a modification of the basic Anchor molecule to the surface can be prevented to the one by the fact that the Anchor exhibits another modification than those, which is used for the coupling of the correctly elongated Splinker molecule to the surface. In the case of identical modifications the free connection places of the matrix can be saturated or blocked by addition the modification of mediating molecule before addition of the Anchor molecule. This can take place with use of the Biotin Streptavidin system for example via addition from soluble biotin. Thus binds Anchor molecule to to the solid matrix coupled, correctly elongated Splinker molecule in consequence hybridizing and/or. Base pairing of the supernatant ends, that a Ligation using suitable ligases follows (D).

[0106] The Ligationsprodukt in such a way received is then split with a Splinker specific restriction enzyme by the type IIS. , The fission product contained received thereby in the projection of the reaction container into a new reaction container is transferred, whereby the correctly elongated Anchor molecule is present here first in solution, but in consequence of the modification to the solid matrix of the new reaction container immobilized (E), permitting a coupling to a solid matrix.

[0107] In a next step a suitable Splinker molecule is in addition-given to the correctly elongated Anchor molecule coupled to the solid matrix. Due to the complementary in each case over-hanging ends of the correctly elongated Anchor molecule and the Splinker molecule these can hybridize with one another. The accumulation product in such a way received can be ligiert then by means of a Ligaseaktivität (F).

[0108] In a last step (H) the Ligationsprodukt with a Anchor specific restriction enzyme of the type IIS, in such a way received, is split and a correctly elongated Splinker molecule in solution is received, which then subject-matter of a Transposition can to be/be able.

[0109] It can recapitulatory be stated that by means of this specific procedure guidance it is ensured that disturbing, D. h. in its sequence correct Oligonukleotide, from the reaction beginning to be removed cannot. The necessity for the distance not correctly elongated Oligonukleotide is relevant for a particularly good yield and correctness of the Oligonukleotids which can be synthesized, which is used in the context of the Sloning method as Anchor or Splinker molecule.

[0110] In principle this method can be used also after each synthesis step. In this case the actual gene synthesis takes place then in solution. Inkorrekte of intermediate products are removed however by the connection to a suitable solid phase from the reaction beginning. Fig. 18 to 20 shows the different steps of such a gene synthesis in solution, with which it concerns a further embodiment of the Sloning method.

[0111] In step (A) becomes here a Anchor molecule, which does not carry modification, which would permit a coupling to a solid phase in this case, with a Splinker molecule ligiert. In this case the Splinker molecule carries a modification, which permits to a coupling to a solid matrix. This reaction takes place in solution, D. h. neither in addition-set Splinker molecule, nor the Ligationsprodukt from the Anchor molecule and the Splinker molecule are first bound to a solid phase (A). In this way a permanent selection is reached on correctly elongated and cut intermediate products. A further advantage of this proceeding is in the fact justified that both as Ligations usually run off as well as restriction reactions in solution with a higher efficiency at the solid phase.

[0112] After inactivation of the ligase by means of the Splinker specific Restriktionsendonuklease by the type IIS one splits. In the consequence an elongated Anchor molecule as well as the Splinker molecule are present again in solution in the reaction beginning. The fission products in such a way received are then transferred into a new reaction container, in which in consequence of the presence of a modification at the Splinker molecule (in the available case a biotin) this binds to the solid matrix. Beside the split Splinker molecule (A) as well as ligierte Splinker molecules are contained in the reaction beginning of the further not cut Ligationsprodukte from step. All three Splinker moleculederivatives bind to the solid phase, not however the elongated Anchor molecule received from step (B), that in solution continue to be present for (C). The projection from step (C), D. h. the elongated Anchor molecule is then transferred into a new reaction container and converted there with a further Splinker molecule, which is more complementary at its ends to the end of the elongated Anchor molecule (E). The new Splinker molecule exhibits again a modification, which permits a coupling to a solid matrix.

[0113] The Ligationsprodukt in such a way received is split after inactivation of the ligase with the Splinker specific Restriktionsendonuklease by the type IIS and supplies thereby far elongated Anchor molecule.

[0114] This proceeding can in principle arbitrarily often be repeated. The special advantage of this embodiment of the Sloning method consists of the fact that a Aufreinigung one is ensured in the Sloning method usable Oligonukleotides. This proceeding can be applied in principle to each level of the Sloning method.

[0115] The Figs. 21 and 22 shows the substantial steps with the synthesis of DNA fragments with internal Methylierung according to the present invention, which can be steps part of the Sloning method. To that extent hereby a further embodiment of the Sloning method is revealed.

[0116] In connection with the description of the figures the term of the Anchors or Anchor molecule designates 21 and 22 in accordance with Oligonukleotid aa) and/or. ba) the Sloning Verfahres and the term of the Splinkers or Splinker molecule in accordance with Oligonukleotid off) and/or. bb) the Sloning method.

[0117] In the context of the synthesis of nucleic acid molecules as for example gene fragments using the Sloning method it was observed that some sequences cannot be synthesized, which is appropriate in it justified that by the Ligation of a Anchor molecule as well as a Splinker molecule a recognition place for a restriction enzyme is trained, that for the restriction enzyme of the Splinker molecule and/or. corresponds to the Anchor molecule and consequence its another splitting event would occur, than for the correct extension of a Anchor and/or. Splinker molecule would be necessary.

[0118] According to invention this restriction of the use of the Sloning method is gone around by the fact that the additional recognition places for either the Anchor, resulting from the specific combination of Anchor and Splinkersequenz, or the Splinker specific restriction enzyme of the type IIS are methyliert and cannot thus not be cut. The recognition sequences within the constant ranges of the Splinker, necessary for the lead-through of the Sloning of method, and/or. Anchor are however methyliert and can still be split therefore.

[0119] Concretely with the fact it is proceeded in such a way that in the context of a sequenziellen Ligation by partly methylierten Splinker molecules from a library it is intended that the 5 ' - overhang sequences ((5 ' - > 3 ') GTC, CTC, TCT) and the following 3 ' - final sequences (AGA, ACG, GAC) are methyliert. In step (A) exhibits a modification basic Anchor molecule in its overhang at the adenosine a Methylierung, permitting a coupling to a solid matrix. The Splinker molecule complementary to the Anchor molecule exhibits a Methylierung in its overhang likewise, in the available case at the cytosine. It is in the context of the present invention that also a Methylierung is possible in other parts than the overhangs, as long as thereby one of the two necessary recognition places for a restriction enzyme is not functionally inactivated by the type IIS.

[0120] After Ligation of the two molecules the Ligationsprodukt represented in (A) is received, which is split after removing the ligase with the Splinker specific Restriktionsendonuklease by the type IIS. In consequence of this cracking elongated the Anchor molecule and now a Methylierung exhibits itself in the two strands of doubling rank. To, the elongated Anchor molecule in such a way received a further Splinker molecule is ligiert, whereby the Splinker molecule leads to the fact that in the Ligationsprodukt beside the Splinker specific restriction enzyme recognition place a further recognition place for the Splinker specific restriction enzyme, contained received in step (C), in the Splinker Molekülteil, is formed. As in (C) thereby beside the recognition place for Eco31I in the Splinker Molekülteil in the Ligationsbereich, indicated in the concrete example, still another further recognition sequence for Eco31I was represented to develop, with which consequence that with use of Eco31I, after removing the ligase, three fission products would develop for typical way. In consequence of the Methylierung second, by the Ligation of the Splinker molecule and the Anchor molecule developed recognition place for Eco31I this is not however accessible for the Splinker specific restriction enzyme. Consequently Eco31I cuts this Ligationsprodukt only Splinker proximally, not however Splinkerdistal. Therefore the Anchor molecule correctly elongated can be used and for the further structure synthesis in the context of the Sloning method.

[0121] Fig. a method points 23 to (between) - product amplification, as it can be accomplished in arbitrary place of the Sloning method. Typical way takes place (between) - product amplification at Ligationsprodukten, which develop in the context of the Sloning method, so for example in the context of the so-called structure synthesis, (i.e. in or repeated going through of the steps aa) to AG) and/or. ba) to bg)) or also in the context of the Transpositionen. Such (between) - product amplification steps are recommended in particular if the concentration one (between) - product it became so low that an efficient lead-through of the following steps is endangered.

[0122] (Between) - product amplification effected via methods of the polymerase nuclear chain reaction well-known as such in the state of the art. It is proceeded in such a way that Anchorund Splinker complementary Oligonukleotidprimer are anelliert to a Ligationsprodukt, how it results in the context of the Sloning method. Preferential way are those primer thereby complementary to the constant part of the Anchor molecule and/or. Splinker molecule, or a part of it. The advantage with this design that primer is that that thereby a pair of primers is sufficient, over independently of developed nucleic acid, i.e. the goal sequence or a part of it, the amplification of the product and/or. to be able to make an intermediate product of the Sloning method. It is however also possible and in the context of the present invention

that those partly or completely bind primer to a range of the Anchor or Splinker molecule, which corresponds to developed nucleic acid. By more complementary it is to be understood with the fact herein that a nucleic acid steps to another nucleic acid by means of base pairing into reciprocal effect. It will be recognized on the part of the specialists that complementary means not necessarily a complete Komplementarität. Rather one or more false base pairings and also exhibit one or more nucleotides no base pairing can be contained.

[0123] With the selection that primer is to be noted that this preferential way should not be more complementary. In consequence its hybridize the primer of preferential way used with this method only with 3-4 nucleotides of the bracket (the constant doppelsträngigen range directly before the loop), the loop range of the Anchor and/or. Splinker molecule as well as the following nucleotides (maximum to the end of the constant part 5 ' - of the overhang of the Anchor molecule and/or. Splinker molecule (A)). After Annelieren of the Anchor and Splinker specific primer an amplification of the internal gene fragments with a thermalstable polymerase takes place, whereby this polymerase of preferential way exhibits a Proofreading function. Those are added to typical way primer in high surplus the reaction beginning. If a far synthesis is intended using (intermediate) the product in such a way amplifizierten, preferential way modified Oligonukleotide is used as primer, which permits a connection of the Oligonukleotide to a solid matrix. The result (between) - product amplification exhibits no more loop structure, which connects the strand and Gegenstrang. Instead the amplifizierte Ligationsprodukt is present as doubling rank structure of two single strands. These molecules called herein also bipartite structure can be likewise used however in the context of the Sloning method as output product.

[0124] It is in the context of the present invention that the different aspects are possible combined for arbitrarily with one another to become to be able and thus a multiplicity of embodiments of the Sloning method.

[0125] The features of the invention revealed in the preceding description, the claims and the designs can be substantial both individually and in arbitrary combination for the implementation of the invention in its different embodiments.